**Lek. Anna Nowak-Szwed**

# Wpływ terapii empagliflozyną na krążące niekodujące RNA związane ze szlakami sirtuinowymi u pacjentów po zawale mięśnia sercowego

**Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne**

Promotor: dr hab. n. med. i n. o zdr. Ceren Eyileten Postula

Katedra i Zakład Farmakologii Doświadczalnej i Klinicznej,
Warszawski Uniwersytet Medyczny


Ceren Eyileten Postula

Anna Nowak-Szwed

Obrona rozprawy doktorskiej przed Radą Dyscypliny Nauk Medycznych
Warszawskiego Uniwersytetu Medycznego

Warszawa 2026 r.

**Słowa kluczowe:**

Empagliflozyna, niekodujące RNA, Ostry zawał mięśnia sercowego, Szlaki sirtuinowe, Farmakogenomika

**Keywords:**

Empagliflozin, non-coding RNA, Acute myocardial infarction, Sirtuin pathways, Pharmacogenomics

**Badania wchodzące w skład niniejszej pracy doktorskiej zostały przeprowadzone w ramach grantów i projektów:**

1. *Farmakogenomika inhibitorów SGLT2 - badanie roli empagliflozyny w epigenetycznej modyfikacji osi niekodujące RNA - sirtuiny - kotransporter sodowo-glukozowy-2 po zawale mięśnia sercowego*. Grant **PRELUDIUM Narodowego Centrum Nauki (NCN)** (2022/45/N/NZ7/0246). Kwota finansowania: 208 866 PLN. **Kierownik grantu: lek. Anna Nowak-Szwed**. Realizacja grantu PRELUDIUM pozwoliła na przeprowadzenie badań w ramach prac 1 i 3.
2. *Empagliflozyna i dapagliflozyna u pacjentów hospitalizowanych z powodu ostrej dekompensacji niewydolności serca*. Grant Agencji Badań Medycznych (ABM) (2019/ABM/01/00037). Kierownik grantu: Prof. dr hab. n. med. Marek Postuła. Realizacja grantu pozwoliła na przeprowadzenie badań w ramach prac 1-3.
3. *Wpływ terapii empagliflozyną na mikroRNA związane ze szlakami sirtuin u pacjentów z pozawałową niewydolnością serca*. Mini-grant studencki, Warszawski Uniwersytet Medyczny (10/M/MG/N/21, 1M9/2/M/MG/N/21). Kwota finansowania: 8 000 PLN. **Kierownik grantu: Anna Nowak-Szwed.** Realizacja mini-grantu studenckiego pozwoliła na uzyskanie wyników wstępnych rozprawy doktorskiej.

Serdeczne podziękowania składam mojej Promotorce, Pani dr hab. n. med. Ceren Eyileten Postula, za nieustanne wsparcie merytoryczne, zaangażowanie oraz zaufanie i motywację na każdym etapie realizacji pracy doktorskiej.

Szczególne wyrazy wdzięczności kieruję do mojego Opiekuna naukowego, Pana Profesora Marka Postuły, którego inspiracja naukowa, wiedza oraz cenne wskazówki stanowiły dla mnie wsparcie w rozwoju naukowym.

Podziękowania składam również Pani Profesor Dagmarze Mirowskiej-Guzel, Kierownik Katedry i Zakładu Farmakologii Doświadczalnej i Klinicznej, za życzliwość, wsparcie oraz stworzenie warunków sprzyjających prowadzeniu działalności naukowej i realizacji niniejszej pracy doktorskiej.

Dziękuję wszystkim współautorom prac naukowych za owocną współpracę oraz wkład w powstanie przedstawionych badań.

Wyrazy najgłębszej wdzięczności kieruję również do mojej rodziny, a w szczególności do mojego Męża za cierpliwość, zrozumienie oraz nieustanne wsparcie i motywację w trakcie realizacji niniejszej pracy. Szczególne podziękowania składam także moim Rodzicom za wiarę we mnie oraz nieustanne wspieranie mojego rozwoju naukowego od najmłodszych lat.

**Wykaz publikacji stanowiących rozprawę doktorską**

1. **Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction.**
   **Nowak-Szwed A.**, Eyileten C., Wicik Z., Ahmadova S., Palatini J., Siller-Matula J., von Lewinski D., Sourij H., Postuła M. *Cardiovascular Diabetology*, 2025; 24:1–20. PMID: 41462250
   **Impact Factor: 10,6; MEiN: 140; Q1**

2. **Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health.**
   Wicik Z., **Nowak-Szwed A.**, Eyileten C., Sourij H., von Lewinski D., Kistkins S., Borkowska J., Postuła M. *npj Systems Biology and Applications*, 2025. PMID: 41274923
   **Impact Factor: 3,5; MEiN: 20; Q1**

3. **MicroRNAs and long non-coding RNAs associated with sirtuin pathways in ischemia/reperfusion injury.**
   **Nowak-Szwed, A.**, Eyileten, C., Wicik, Z., Ahmadova, S., von Lewinski, D., Sourij, H. & Postula, M., Cardiovascular Drugs and Therapy, 2026. PMID: 41849042.
   **Impact Factor: 3,1; MEiN: 100; Q2**

**Łącznie:**
**IF: 17,2**
**MEiN: 260**

**Wykaz wszystkich publikacji**

| Rodzaj publikacji | Liczba | Impact Factor | Punkty MEiN |
|---|---|---|---|
| **Prace włączone do rozprawy doktorskiej** | **3** | **17,2** | **260** |
| **Prace, które nie zostały włączone do rozprawy doktorskiej** | **16** | **51,3** | **1195** |
| **Razem** | **19** | **68,5** | **1455** |

**Wykaz abstraktów prezentowanych na konferencjach międzynarodowych przez lek. Annę Nowak-Szwed**

1. **Nowak-Szwed A.**, Eyileten C., Wicik Z., Ahmadova S., Siller-Matula J., von Lewinski D., Sourij H., Postula M. ***The predictive value of sirtuins and miRNAs in cardiac recovery after acute myocardial infarction treated with empagliflozin*.** 85th Scientific Sessions, American Diabetes Association Congress, Czerwiec 20–23, 2025, Chicago, IL, USA. Sesja plakatowa. Finansowanie: PRELUDIUM NCN (2022/45/N/NZ7/0246). Grant ABM (2019/ABM/01/00037). *Diabetes* 20 June 2025; 74 (Suppl 1): 906–P. https://doi.org/10.2337/db25-906-P
2. **Nowak A.**, Wicik Z., Eyileten C., Ahmadova S., Siller-Matula J., von Lewinski D., Sourij H., Postula M. ***Empagliflozin's heart shield: unlocking sirtuins and ncRNA regulators for myocardial infarction recovery.*** 60th Annual Meeting of the European Association for the Study of Diabetes (EASD), Wrzesień 9–13, 2024, Madryt, Hiszpania. Sesja krótkich prac ustnych. Finansowanie: PRELUDIUM NCN (2022/45/N/NZ7/0246). Grant ABM (2019/ABM/01/00037). *Diabetologia* 67 (Suppl 1), 1–593 (2024). https://doi.org/10.1007/s00125-024-06226-0
3. **Nowak A.**, Wicik Z., Eyileten C., Ahmadova S., Siller-Matula J., von Lewinski D., Sourij H., Postula M. ***Cardioprotective effect of empagliflozin and modification of sirtuins and their noncoding RNA regulators in patients with myocardial infarction.*** 84th Scientific Sessions, American Diabetes Association Congress, Czerwiec 21–24, 2024, Orlando, FL, USA. Sesja prac ustnych. Finansowanie: PRELUDIUM NCN (2022/45/N/NZ7/0246). Grant ABM (2019/ABM/01/00037). *Diabetes* 14 June 2024; 73 (Suppl 1): 141–OR. https://doi.org/10.2337/db24-141-OR
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# Spis treści

## 1. Wykaz stosowanych skrótów

| Skrót | Rozwinięcie w j. angielskim | Rozwinięcie w j. polskim |
|---|---|---|
| AMI | Acute Myocardial Infarction | Ostry zawał mięśnia sercowego |
| AUC | Area Under the Curve | Pole pod krzywą |
| CVD | Cardiovascular Diseases | Choroby układu sercowo-naczyniowego |
| GCK | Glucokinase | Glukokinaza |
| GIP | Glucose-dependent Insulinotropic Polypeptide | Glukozozależny peptyd insulinotropowy |
| GLP-1 | Glucagon-like Peptide-1 | Glukagonopodobny peptyd-1 |
| GLP-1 RA | Glucagon-like Peptide-1 Receptor Agonist | Agonista receptora glukagonopodobnego peptydu-1 |
| HF | Heart Failure | Niewydolność serca |
| I/R | Ischemia/Reperfusion | Niedokrwienie/reperfuzja |
| lncRNA | Long Non-coding RNA | Długie niekodujące RNA |
| MI | Myocardial Infarction | Zawał mięśnia sercowego |
| miRNA | MicroRNA | MikroRNA |
| $NAD^+$ | Nicotinamide Adenine Dinucleotide | Dinukleotyd nikotynoamidoadeninowy |
| ncRNA | Non-coding RNA | Niekodujące RNA |
| qRT-PCR | Quantitative Real-Time Polymerase Chain Reaction | Ilościowa reakcja łańcuchowa polimerazy w czasie rzeczywistym |
| SGLT2 | Sodium-Glucose Cotransporter 2 | Kotransporter sodowo-glukozowy typu 2 |
| SIRT | Sirtuin | Sirtuina |

## 2. Streszczenie w języku polskim

### Wpływ terapii empagliflozyną na krążące niekodujące RNA związane ze szlakami sirtuinowymi u pacjentów po zawale mięśnia sercowego

Zawał mięśnia sercowego, będący jedną z głównych manifestacji chorób układu sercowo-naczyniowego, prowadzi do trwałego uszkodzenia mięśnia sercowego, przebudowy lewej komory i w konsekwencji do rozwoju niewydolności serca o etiologii niedokrwiennej. Jednym z kluczowych mechanizmów odpowiedzialnych za uszkodzenie mięśnia sercowego po zawale jest uraz niedokrwienno-reperfuzyjny (*ischemia/reperfusion injury*, I/R), który obejmuje złożoną sieć procesów molekularnych, takich jak stres oksydacyjny, dysfunkcja mitochondrialna, aktywacja procesów zapalnych oraz apoptoza kardiomiocytów. Wśród regulatorów molekularnych uczestniczących w tych procesach szczególną rolę odgrywają sirtuiny (SIRT), które należą do rodziny enzymów zależnych od dinukleotydu nikotynoamidoadeninowego ($NAD^+$). Ich rola polega na regulacji metabolizmu komórkowego oraz mechanizmów epigenetycznych poprzez wpływ na stres oksydacyjny, starzenie komórkowe, funkcję mitochondriów oraz modulację szlaków zapalnych aktywowanych w przebiegu uszkodzenia mięśnia sercowego.

Równocześnie, coraz więcej badań wskazuje, że regulacja ekspresji genów w chorobach układu sercowo-naczyniowego zależy między innymi od działania niekodujących RNA (*non-coding RNA*, ncRNA), w tym mikroRNA (*microRNA,* miRNA) oraz długich niekodujących RNA (*long non-coding RNA*, lncRNA). Cząsteczki te stanowią regulatory posttranskrypcyjne zdolne do modulowania ekspresji białek zaangażowanych w procesy związane z uszkodzeniem mięśnia sercowego oraz przebudową mięśnia sercowego i coraz więcej dowodów naukowych wskazuje, że mogą one wpływać na patofizjologię chorób układu sercowo-naczyniowego.

Jednocześnie współczesna farmakoterapia chorób kardiometabolicznych uległa znacznemu postępowi dzięki nowym lekom stosowanym do tej pory w leczeniu cukrzycy typu 2. Inhibitory kotransportera sodowo-glukozowego typu 2 (SGLT2) wykazują działanie kardioprotekcyjne niezależnie od ich wpływu na gospodarkę węglowodanową. W badaniach klinicznych wykazano, że leki te zmniejszają ryzyko zdarzeń sercowo-naczyniowych oraz poprawiają rokowanie u pacjentów z cukrzycą typu 2 i/lub wysokim ryzykiem sercowo-naczyniowym. Pomimo licznych badań klinicznych mechanizmy molekularne odpowiedzialne za ich działanie ochronne na układ sercowo-naczyniowy pozostają w dużej mierze niewyjaśnione. Jedną z hipotez stanowi modulacja szlaków

SIRT. Dodatkowo, odpowiedź na leczenie różni się między pacjentami, co stanowi istotne wyzwanie w praktyce klinicznej. Zrozumienie tych mechanizmów może przyczynić się do identyfikacji nowych celów terapeutycznych oraz rozwoju spersonalizowanych strategii leczenia.

Głównym celem niniejszej rozprawy doktorskiej była ocena roli krążących ncRNA związanych ze szlakami sygnałowymi SIRT u pacjentów po zawale mięśnia sercowego leczonych inhibitorem SGLT2 - empagliflozyną, oraz analiza ich użyteczności jako nowych biomarkerów predykcji odpowiedzi na lek. Praca łączy podejście kliniczne, molekularne i bioinformatyczne w celu identyfikacji szlaków regulacyjnych empagliflozyna–ncRNA–SIRT zaangażowanych w patofizjologię zawału mięśnia sercowego.

Centralną część rozprawy stanowi praca oryginalna analizująca molekularne efekty terapii empagliflozyną u pacjentów po zawale mięśnia sercowego. W pierwszym etapie badań przeprowadzono identyfikację miRNA związanych ze szlakami regulowanymi przez SIRT z wykorzystaniem narzędzi bioinformatycznych. Uzyskane wyniki analiz *in silico* stanowiły podstawę do opracowania wniosku grantowego, który został złożony w konkursie PRELUDIUM Narodowego Centrum Nauki i uzyskał finansowanie przy pierwszej aplikacji (nr 2022/45/N/NZ7/0246). Następnie przeprowadzono walidację ekspresji wytypowanych miRNA oraz SIRT w próbkach osocza pochodzących od pacjentów uczestniczących w randomizowanym badaniu klinicznym *Empagliflozin in acute myocardial infarction: the EMMY trial*, w którym pacjenci otrzymywali inhibitor SGLT2 - empagliflozynę lub placebo. Ekspresję wybranych miRNA oraz genów kodujących sirtuiny (SIRT1–SIRT7) oceniono metodą qRT-PCR przed włączeniem terapii oraz po 26 tygodniach leczenia wśród 227 pacjentów. Wykazano, że terapia inhibitorem SGLT2 istotnie moduluje ekspresję SIRT oraz regulatorowych miRNA. Po 26 tygodniach leczenia obserwowano obniżoną ekspresję SIRT4 ($p=0.018$) oraz podwyższoną ekspresję SIRT6 ($p=0.006$) w porównaniu z grupą placebo. Ponadto wykazano, że wyjściowa ekspresja SIRT2 i SIRT4, a także miR-182-5p i miR-302a-3p może stanowić niezależny panel predykcyjny odpowiedzi na leczenie empagliflozyną, biorąc pod uwagę zmianę frakcji wyrzutowej lewej komory serca po 26 tygodniach terapii (AUC: 0.890; czułość 81%; swoistość 90%). Wyniki te wskazują, że krążące ncRNA oraz szlaki molekularne związane z sirtuinami mogą stanowić potencjalne biomarkery odpowiedzi na leczenie inhibitorami SGLT2 u pacjentów po zawale mięśnia sercowego. Badanie wskazuje również na możliwe epigenetyczne mechanizmy działania empagliflozyny oraz podkreśla znaczenie biomarkerów

molekularnych w rozwoju spersonalizowanych strategii leczenia chorób układu sercowo-naczyniowego.

Druga publikacja, o charakterze bioinformatycznym, rozszerza kontekst molekularny rozprawy poprzez analizę *in silico*. W pracy tej wykazano, że nowoczesne terapie kardiometaboliczne działają w obrębie częściowo wspólnych sieci regulacyjnych obejmujących procesy związane z gospodarką glukozową, metabolizmem energetycznym, odpowiedzią zapalną, funkcją naczyń oraz odpowiedzią na stres komórkowy. Wyniki te stanowią systemowe tło interpretacyjne dla obserwacji uzyskanych w części klinicznej.

Trzecią część rozprawy stanowi praca przeglądowa poświęcona roli ncRNA regulujących szlaki sygnałowe SIRT w uszkodzeniu niedokrwienno-reperfuzyjnym mięśnia sercowego. W pracy tej podsumowano aktualną wiedzę eksperymentalną dotyczącą regulacji SIRT przez miRNA oraz lncRNA, a także ich wpływu na kluczowe procesy komórkowe związane z uszkodzeniem mięśnia sercowego. Analiza dostępnej literatury wskazuje, że cząsteczki ncRNA modulują liczne procesy biologiczne, w tym apoptozę, stres oksydacyjny, dysfunkcję mitochondriów oraz odpowiedź zapalną, które odgrywają istotną rolę w patofizjologii uszkodzenia niedokrwienno-reperfuzyjnego. Praca podkreśla również potencjał ncRNA jako celów terapeutycznych oraz nowych biomarkerów prognostycznych i diagnostycznych w zawale mięśnia sercowego.

Podsumowując, przedstawiona rozprawa dostarcza nowych danych dotyczących znaczenia osi ncRNA-SIRT w odpowiedzi mięśnia sercowego na leczenie empagliflozyną po zawale mięśnia sercowego. Uzyskane wyniki podkreślają znaczenie epigenetycznych mechanizmów regulacyjnych w chorobach układu sercowo-naczyniowego oraz wskazują na potencjalną rolę krążących ncRNA jako biomarkerów odpowiedzi na leczenie. Praca ta pokazuje również, że integracja badań molekularnych, klinicznych i analiz bioinformatycznych sprzyja lepszemu zrozumieniu złożonych mechanizmów patofizjologicznych chorób kardiometabolicznych oraz może wspierać identyfikację nowych celów terapeutycznych i rozwój strategii medycyny spersonalizowanej, ukierunkowanych na poprawę rokowania pacjentów.

## 3. Streszczenie w jezyku angielskim

### Effects of empagliflozin therapy on circulating non-coding RNAs associated with sirtuin pathways in patients after myocardial infarction

Myocardial infarction (MI), one of the major clinical manifestations of cardiovascular disease (CVD), leads to permanent myocardial damage, left ventricular remodeling, and consequently to the development of heart failure (HF) of ischemic etiology. One of the key mechanisms responsible for myocardial injury following infarction is ischemia/reperfusion (I/R) injury, which involves a network of molecular processes, including oxidative stress, mitochondrial dysfunction, activation of inflammatory pathways, and cardiomyocyte apoptosis. Among the molecular regulators involved in these processes are sirtuins (SIRT), which belong to the family of nicotinamide adenine dinucleotide ($NAD^+$)-dependent enzymes. Their role involves the regulation of cellular metabolism and epigenetic mechanisms through effects on oxidative stress, cellular senescence, mitochondrial function, and the modulation of inflammatory pathways activated during myocardial injury.

An increasing body of evidence indicates that gene expression in CVD is extensively regulated by non-coding RNAs (ncRNAs), including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). These molecules act as post-transcriptional regulators capable of modulating the expression of proteins involved in myocardial injury and cardiac remodeling, and growing evidence indicates that they may contribute to the pathophysiology of CVD.

In parallel, modern pharmacotherapy for cardiometabolic diseases has evolved substantially with the introduction of novel drug classes originally developed for the management of type 2 diabetes (T2D). Sodium-glucose cotransporter 2 (SGLT2) inhibitors have demonstrated significant cardioprotective effects independent of their glucose-lowering properties. Clinical studies have shown that these therapies reduce the risk of CV events and improve outcomes in patients with diabetes and those at high CV risk. However, despite robust clinical evidence, the molecular mechanisms underlying their CV protective effects remain not fully understood; one hypothesis is that SGLT2 inhibitors modulate SIRT pathways. Moreover, interindividual variability in treatment response remains a major challenge in clinical settings. A deeper understanding of these mechanisms may facilitate the identification of novel therapeutic targets and support the development of personalized treatment strategies.

Therefore, the main aim of my doctoral thesis was to evaluate the role of circulating ncRNAs associated with SIRT pathways in patients after MI treated with SGLT2 inhibitor - empagliflozin, and to investigate their utility as novel predictive biomarkers of drug response. This work integrates clinical, molecular, and bioinformatic approaches to identify the empagliflozin-ncRNA-SIRT axis involved in MI-related processes.

The central part of the thesis consists of an original study analyzing the molecular effects of empagliflozin in patients after MI. In the first stage of the study, miRNAs associated with SIRT pathways were identified using bioinformatic tools. The results of these *in silico* analyses enabled the development of a grant proposal submitted to the PRELUDIUM competition of the Polish National Science Centre, which was funded on the first submission (2022/45/N/NZ7/0246). Subsequently, the expression of top miRNAs and SIRT was validated in plasma samples obtained from patients after MI participating in the randomized clinical trial: *Empagliflozin in acute myocardial infarction: the EMMY trial*, in which patients received empagliflozin or placebo. The expression of selected miRNAs and SIRT1-7 was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) before treatment initiation and after 26 weeks of therapy in 227 patients. After 26 weeks of treatment, decreased SIRT4 expression ($p=0.018$) and increased SIRT6 expression ($p=0.006$) compared with the placebo group were observed. Furthermore, baseline expression levels of SIRT2 and SIRT4, together with miR-182-5p and miR-302a-3p, demonstrated high predictive accuracy as a panel for empagliflozin response, as assessed by changes in left ventricular ejection fraction (AUC: 0.890; 81% sensitivity; 90% specificity). These findings highlight the potential of these biomarkers for stratifying responders and non-responders to empagliflozin in patients after MI. The study also indicates possible epigenetic mechanisms underlying the effects of empagliflozin and highlights the importance of molecular biomarkers in developing personalized therapeutic strategies for CVD.

The second publication is an original bioinformatic study that broadens the molecular context of the dissertation through *in silico* analysis. This study demonstrated that modern cardiometabolic therapies act within partly overlapping regulatory networks involving glucose homeostasis, energy metabolism, inflammatory responses, vascular function, and cellular stress responses. These findings provide a system-level interpretative background for the observations obtained in the clinical study.

The third part of the thesis consists of a review article focused on the role of ncRNAs regulating SIRT signaling pathways in myocardial I/R injury. This publication summarizes current

experimental evidence on the regulation of SIRT by miRNAs and lncRNAs and their impact on key cellular processes involved in I/R. The literature analysis indicates that ncRNAs can modulate multiple biological processes, including apoptosis, oxidative stress, mitochondrial dysfunction, and inflammatory response, which are critical in the pathophysiology of I/R injury, presenting them as possible treatment targets and novel prognostic and diagnostic biomarkers in MI.

In summary, the present dissertation provides novel data on the importance of the ncRNA–SIRT axis in myocardial response to empagliflozin treatment after MI. The obtained results underscore the importance of epigenetic regulatory mechanisms in CVD and point to the potential role of circulating ncRNAs as biomarkers of drug response. This work also shows that integrating molecular, clinical, and bioinformatic research contributes to a better understanding of the complex pathophysiological mechanisms of cardiometabolic diseases and may support the identification of new therapeutic targets and the development of personalized medicine strategies aimed at improving patient prognosis.

## 4. Wstęp uzasadniający połączenie wskazanych publikacji w jeden cykl, jak i komentujący osiągnięcie naukowe kandydata na tle dotychczasowego stanu wiedzy

Choroby układu sercowo-naczyniowego pozostają główną przyczyną zgonów na świecie i stanowią jedno z najpoważniejszych wyzwań współczesnej medycyny. Pomimo znaczącego postępu w zakresie diagnostyki oraz leczenia choroby niedokrwiennej serca, ostry zawał mięśnia sercowego nadal jest jednym z najważniejszych czynników prowadzących do rozwoju niewydolności serca oraz zwiększonej śmiertelności w populacji dorosłych (Frantz et al., 2022). Wprowadzenie nowoczesnych metod leczenia reperfuzyjnego, w szczególności przezskórnych interwencji wieńcowych, istotnie poprawiło rokowanie pacjentów w ostrej fazie zawału. Jednakże, pomimo skutecznego przywrócenia przepływu w tętnicach wieńcowych u wielu pacjentów dochodzi do wtórnego uszkodzenia mięśnia sercowego wynikającego z urazu niedokrwienno-reperfuzyjnego (*ischemia/reperfusion injury*, I/R) (Harrington et al., 2022). To właśnie ten proces, obejmujący między innymi stres oksydacyjny, dysfunkcję mitochondriów, aktywację szlaków zapalnych oraz apoptozę kardiomiocytów, odgrywa istotną rolę w przebudowie lewej komory i dalszym rozwoju niewydolności serca po zawale mięśnia sercowego (Frank et al., 2012, Welt et al., 2024).

W ostatnich latach coraz większą uwagę poświęca się molekularnym mechanizmom regulującym odpowiedź mięśnia sercowego na uszkodzenie niedokrwienno-reperfuzyjne. Szczególne znaczenie przypisuje się SIRT, czyli rodzinie enzymów zależnych od NAD+, uczestniczących w regulacji metabolizmu komórkowego, odpowiedzi na stres oksydacyjny, procesów zapalnych, funkcji mitochondriów, starzenia komórkowego oraz przeżycia komórek (Houtkooper et al., 2012; Wu et al., 2022). Dotychczas zidentyfikowano siedem izoform SIRT u ludzi (SIRT1–SIRT7), które różnią się lokalizacją komórkową oraz pełnionymi funkcjami biologicznymi. W układzie sercowo-naczyniowym SIRT stanowią istotne regulatory adaptacji do stresu metabolicznego i oksydacyjnego. Dotychczasowe badania eksperymentalne wskazują, że część izoform SIRT może wykazywać działanie kardioprotekcyjne w warunkach niedokrwienia i reperfuzji, jednak ich rola pozostaje złożona i zależna od kontekstu biologicznego, rodzaju modelu oraz etapu uszkodzenia mięśnia sercowego. Z tego względu dalsze badania nad szlakami sirtuinowymi są uzasadnione zarówno z punktu widzenia patofizjologii zawału mięśnia sercowego, jak i potencjalnych zastosowań diagnostycznych i terapeutycznych (Sola-Sevilla et al., 2025; Wu et al., 2022).

Równolegle z rozwojem badań nad sirtuinami coraz większe znaczenie przypisuje się mechanizmom epigenetycznym, w tym regulacji ekspresji genów przez ncRNA. Do najlepiej poznanych klas tych cząsteczek należą miRNA oraz lncRNA (Jakubik et al., 2021). MiRNA są krótkimi cząsteczkami RNA, średnio o długości 23 nukleotydów, regulującymi ekspresję genów na poziomie post-transkrypcyjnym poprzez wiązanie się z docelowymi sekwencjami mRNA i hamowanie translacji lub ich degradację (Zhou et al., 2018). Z kolei lncRNA stanowią zróżnicowaną grupę cząsteczek RNA o długości powyżej 200 nukleotydów, które mogą regulować ekspresję genów poprzez m.in. oddziaływania z białkami, innymi niekodującymi RNA oraz chromatyną (Mattick et al., 2023).

Coraz więcej badań wskazuje, że ncRNA odgrywają istotną rolę w patofizjologii chorób układu sercowo-naczyniowego. Wykazano, że liczne miRNA uczestniczą w regulacji procesów zapalnych, stresu oksydacyjnego, angiogenezy oraz przebudowy mięśnia sercowego (Zhou et al., 2018). Szczególnie interesującym obszarem badań jest interakcja pomiędzy ncRNA, a szlakami sygnałowymi związanymi z SIRT. Badania przedkliniczne na modelach komórkowych i zwierzęcych sugerują, że miRNA mogą regulować ekspresję poszczególnych izoform sirtuin, wpływając tym samym na przeżycie kardiomiocytów w warunkach niedokrwienia (Welt et al., 2024). Z kolei niektóre lncRNA mogą modulować aktywność miRNA poprzez mechanizm tzw. "gąbki molekularnej" (ang. *sponging*), co prowadzi do wtórnej regulacji ekspresji genów SIRT (Liu et al., 2022). Tym samym istnieje wyraźna luka badawcza pomiędzy wiedzą przedkliniczną a możliwością wykorzystania ncRNA i sirtuin jako biomarkerów lub celów terapeutycznych u pacjentów po zawale mięśnia sercowego.

Równolegle do postępu w badaniach molekularnych w ostatnich latach nastąpił dynamiczny rozwój nowych terapii chorób kardiometabolicznych. Szczególne znaczenie mają inhibitory SGLT2, początkowo stosowane w leczeniu cukrzycy typu 2, które wykazały korzystny wpływ na układ sercowo-naczyniowy. W badaniach eksperymentalnych i klinicznych sugerowano, że ich działanie wykracza poza efekt hipoglikemizujący i może obejmować poprawę metabolizmu mięśnia sercowego, ograniczenie stresu oksydacyjnego, modulację procesów zapalnych, wpływ na włóknienie oraz ochronę przed uszkodzeniem niedokrwienno-reperfuzyjnym. Jedną z najbardziej interesujących hipotez mechanistycznych jest udział szlaków sirtuinowych w kardioprotekcyjnym działaniu inhibitorów SGLT2 (Badve et al., 2025; Patel et al., 2024).

Mechanizmy molekularne inhibitorów SGLT2 odpowiedzialne za obserwowane korzyści sercowo-naczyniowe nie zostały jednak w pełni wyjaśnione. Sugeruje się, że mogą one obejmować zmiany w metabolizmie energetycznym mięśnia sercowego, poprawę funkcji śródbłonka, redukcję stresu oksydacyjnego oraz modulację procesów zapalnych (Lopaschuk & Verma, 2020; Reed et al., 2018). Coraz więcej badań wskazuje również na możliwy udział mechanizmów epigenetycznych w działaniu tych leków (Saravana Kumar et al., 2025; Zhao et al., 2026). Jedną z hipotez jest też wpływ inhibitorów SGLT2 na aktywację szlaków sygnałowych związanych z sirtuinami, co może prowadzić do zwiększenia odporności komórek na stres metaboliczny oraz poprawy funkcji mitochondriów (Packer, 2020).

Pomimo rosnącej liczby badań eksperymentalnych dotyczących tych mechanizmów, nadal istnieje istotna luka w wiedzy na temat ich znaczenia u ludzi, szczególnie w kontekście pacjentów po zawale mięśnia sercowego. W związku z powyższym, głównym celem przedstawionej rozprawy doktorskiej jest ocena roli krążących niekodujących RNA związanych ze szlakami sygnałowymi SIRT u pacjentów po zawale mięśnia sercowego leczonych empagliflozyną oraz analiza ich potencjału jako nowych biomarkerów predykcji odpowiedzi na leczenie. Praca została zaplanowana jako cykl trzech komplementarnych publikacji, łączących podejście kliniczne, bioinformatyczne i mechanistyczne.

Centralny element rozprawy stanowi pierwsza publikacja, będąca pracą oryginalną o charakterze kliniczno-translacyjnym, w której oceniono wartość predykcyjną miRNA i SIRT jako markerów odpowiedzi na terapię empagliflozyną u pacjentów po zawale mięśnia sercowego, mierzoną zmianą frakcji wyrzutowej lewej komory. W badaniu tym wykorzystano próbki osocza pochodzące od pacjentów uczestniczących w randomizowanym badaniu klinicznym EMMY (*Empagliflozin in acute myocardial infarction: the EMMY trial)* (Tripolt et al., 2020; von Lewinski et al., 2022).

Na podstawie analiz bioinformatycznych wytypowano miRNA związane ze szlakami sirtuinowymi, a następnie oceniono ich ekspresję oraz ekspresję genów SIRT1–SIRT7 metodą qRT-PCR. Następnie przeprowadzono obszerną analizę statystyczną integrując dane kliniczne i uzyskane wyniki laboratoryjne. Wykazano, że po 26 tygodniach leczenia empagliflozyną ekspresja SIRT6 była wyższa ($p=0.006$), a ekspresja SIRT4 niższa w porównaniu z grupą placebo ($p=0.018$). Ponadto wykazano, że wyjściowa ekspresja SIRT2 i SIRT4, a także miR-182-5p i miR-302a-3p może stanowić niezależny panel predykcyjny odpowiedzi na leczenie empagliflozyną, biorąc pod uwagę

zmianę frakcji wyrzutowej lewej komory serca po 26 tygodniach terapii (AUC: 0.890; czułość 81%; swoistość 90%) (Nowak-Szwed et al., 2025). Wynik ten stanowi najważniejsze osiągnięcie przedstawionego cyklu, ponieważ po raz pierwszy wskazuje na potencjalną wartość osi ncRNA-SIRT jako biomarkera predykcyjnego odpowiedzi na empagliflozynę u pacjentów po zawale mięśnia sercowego. Dodatkowo, wyniki te dostarczają istotnych informacji na temat modyfikacji epigenetycznych związanych ze stosowaniem inhibitorów SGLT2 oraz sugerują, że charakterystyka genomiczna może przyczynić się do rozwoju farmakoterapii personalizowanej (Nowak-Szwed et al., 2025).

Druga publikacja oryginalna obejmuje analizę bioinformatyczną i została włączona do cyklu jako praca rozszerzająca kontekst molekularny nowoczesnych terapii kardiometabolicznych. W badaniu tym przeanalizowano sieci interakcji związane z działaniem inhibitorów SGLT2 oraz leków inkretynowych w chorobach układu sercowo-naczyniowego. Analiza sieci interakcji pozwoliła zidentyfikować procesy związane z regulacją metabolizmu, odpowiedzią zapalną i funkcją układu sercowo-naczyniowego. Wyniki pokazują, że współczesne terapie kardiometaboliczne działają w obrębie częściowo wspólnych sieci regulacyjnych, obejmujących procesy istotne również dla odpowiedzi mięśnia sercowego po zawale. Tym samym publikacja ta stanowi szersze systemowe tło interpretacyjne dla wyników części klinicznej i pozwala zaznaczyć rolę empagliflozyny w szerszym kontekście molekularnych mechanizmów kardioprotekcyjnych (Wicik, Nowak-Szwed et al., 2025).

Trzecia publikacja, o charakterze przeglądowym, podsumowuje aktualny stan wiedzy dotyczący roli ncRNA regulujących szlaki sirtuinowe w uszkodzeniu niedokrwienno-reperfuzyjnym mięśnia sercowego (Nowak-Szwed et al., 2026). Praca stanowi kompleksową syntezę aktualnego stanu wiedzy dotyczącego interakcji pomiędzy miRNA, lncRNA oraz SIRT w kontekście patofizjologii zawału mięśnia sercowego. Ta unikalna, pierwsza o takiej tematyce praca przeglądowa pozwoliła zidentyfikować i uporządkować osie regulacyjne ncRNA-SIRT zaangażowane w kontrolę procesów apoptotycznych, regulację funkcji mitochondriów oraz modulację zapalenia w odpowiedzi na uszkodzenie mięśnia sercowego. Większość dotychczasowych badań w tym obszarze opierała się na modelach zwierzęcych oraz eksperymentach *in vitro*, co wskazuje na istotną lukę w badaniach z udziałem pacjentów. Jej cel w ramach rozprawy polega na dostarczeniu biologicznego i mechanistycznego uzasadnienia dla wyników uzyskanych w badaniu klinicznym. Jednocześnie praca ta podkreśla, że dotychczasowe dane mają głównie charakter przedkliniczny, co jeszcze silniej

uwidacznia translacyjną wartość pierwszej publikacji (Nowak-Szwed et al., 2025; Nowak-Szwed et al., 2026).

Połączenie tych trzech publikacji w jeden cykl jest uzasadnione ich komplementarnym charakterem oraz wspólną osią badawczą. Wszystkie trzy prace odnoszą się do molekularnych mechanizmów kardioprotekcji, ze szczególnym uwzględnieniem osi ncRNA–SIRT i jej znaczenia w odpowiedzi mięśnia sercowego na uszkodzenie oraz leczenie. Publikacja pierwsza dostarcza danych klinicznych i biomarkerowych, publikacja druga poszerza perspektywę o analizę systemowych sieci regulacyjnych nowoczesnych terapii kardiometabolicznych, natomiast publikacja trzecia dostarcza mechanistycznego uzasadnienia biologicznego. Razem tworzą spójny program badawczy, którego celem jest lepsze zrozumienie mechanizmów działania empagliflozyny po zawale mięśnia sercowego oraz identyfikacja nowych biomarkerów mogących wspierać rozwój terapii spersonalizowanej.

Przedstawiony cykl publikacji stanowi istotny wkład w rozwój badań nad molekularnymi mechanizmami chorób układu sercowo-naczyniowego oraz rolą regulatorów epigenetycznych w patofizjologii zawału mięśnia sercowego. Najważniejszym osiągnięciem pracy jest wykazanie, że wybrane sirtuiny i regulatorowe miRNA mogą mieć znaczenie predykcyjne dla odpowiedzi na empagliflozynę w populacji pacjentów po ostrym zawale mięśnia sercowego. Wyniki te wspierają hipotezę, że epigenetyczne mechanizmy regulacyjne, w tym oś ncRNA-SIRT, mogą odgrywać istotną rolę w procesach naprawczych i przebudowie mięśnia sercowego, a w przyszłości mogą znaleźć zastosowanie w stratyfikacji pacjentów i rozwoju bardziej spersonalizowanych strategii terapeutycznych umożliwiając lepszy dobór terapii oraz poprawę rokowania i jakości życia pacjentów.

## 5. Założenia i cel pracy

Głównym celem pracy doktorskiej była ocena roli krążących ncRNA związanych ze szlakami sygnałowymi SIRT u pacjentów po zawale mięśnia sercowego leczonych inhibitorem SGLT2 - empagliflozyną, oraz analiza ich użyteczności jako nowych biomarkerów predykcji odpowiedzi na lek. Założenie to wynikało z danych klinicznych wskazujących na kardioprotekcyjne działanie inhibitorów SGLT2 oraz danych przedklinicznych sugerujących udział SIRT i ncRNA w regulacji procesów związanych z urazem niedokrwienno-reperfuzyjnym. Praca integruje aspekty kliniczne, molekularne oraz bioinformatyczne w celu identyfikacji szlaków empagliflozyna-ncRNA-SIRT zaangażowanych w procesy związane z zawałem mięśnia sercowego, ze szczególnym uwzględnieniem ich znaczenia klinicznego i potencjalnych zastosowań translacyjnych.

Cele szczegółowe obejmowały:

1. identyfikację miRNA potencjalnie związanych z regulacją szlaków SIRT z wykorzystaniem narzędzi bioinformatycznych;
2. ocenę ekspresji wybranych miRNA oraz genów SIRT1–SIRT7 w próbkach osocza pacjentów po zawale mięśnia sercowego uczestniczących w badaniu EMMY w momencie włączenia do badania i po 26 tygodniach leczenia;
3. ocenę wartości predykcyjnej wybranych cząsteczek względem zmiany funkcji skurczowej lewej komory po 26 tygodniach terapii empagliflozyną;
4. analizę szerszego kontekstu molekularnego nowoczesnych terapii kardiometabolicznych poprzez ocenę sieci interakcji inhibitorów SGLT2
5. podsumowanie aktualnej wiedzy na temat roli osi ncRNA-SIRT w uszkodzeniu niedokrwienno-reperfuzyjnym mięśnia sercowego.

## 6. Kopie opublikowanych prac

### 6.1. Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction

Nowak-Szwed *et al. Cardiovascular Diabetology* (2025) 24:463
https://doi.org/10.1186/s12933-025-03013-y

Cardiovascular Diabetology

RESEARCH Open Access

# Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction

Anna Nowak-Szwed[1,2,3], Ceren Eyileten[1,4], Zofia Wicik[1,5], Sara Ahmadova[1,3], Jeff Palatini[4], Jolanta Siller-Matula[6], Dirk von Lewinski[7], Harald Sourij[8] and Marek Postula[1*]

**Abstract**

**Background** Sodium-glucose cotransporter-2 (SGLT2) inhibitors, primarily used to treat type 2 diabetes, exhibit cardioprotective effects by improving myocardial energy metabolism, reducing oxidative stress, and modulating inflammation and fibrosis, which are critical in the context of acute myocardial infarction (AMI). Our research aims to explore the molecular mechanisms of SGLT2 inhibitors, with a focus on their influence on non-coding RNAs through sirtuins pathways, to identify novel biomarkers and therapeutic strategies for preventing heart failure following AMI.

**Methods** We identified microRNAs (miRNAs) that play a role in sirtuin pathways in AMI. We validated the expressions of precisely selected miRNAs along with sirtuin gene expressions (SIRT1-7) in a total of 227 patients with samples from baseline and after 26-week of either placebo or empagliflozin treatment by qRT-PCR. We also performed SHAP analysis of clinical data and miRNAs target predictions and advanced enrichment analyses.

**Results** Empagliflozin treatment significantly modulated sirtuin and miRNA expression, with higher SIRT6 ($p < 0.001$) and lower SIRT4 ($p = 0.018$) expression compared to placebo after 26 weeks.($p$ ($p$ In contrast, patients in the placebo group showed a reduction in SIRT6 expression ($p = 0.006$). Patients were divided according to the change in LVEF (ΔLVEF) between baseline and 26-weeks, using a cut-off of 11%. This threshold was derived from the third quartile distribution in the empagliflozin group. Baseline SIRT2 and SIRT4 levels independently predicted a ΔLVEF < 11% improvement (AUC: 0.806 and 0.765, respectively; both $p < 0.01$), as did miR-182-5p and miR-302a-3p (AUC: 0.716 and 0.757; both $p < 0.01$). A combined biomarker panel including SIRT2, SIRT4, miR-182-5p, and miR-302a-3p demonstrated superior predictive accuracy for ΔLVEF < 11% after 26-weeks of empagliflozin treatment (cross-validated AUC: 0.890; 81% sensitivity; 90% specificity). This association remained significant after multivariate adjustment for age, sex, hypertension, BMI, and ezetimibe treatment (OR: 18.70; 95% CI: 5.78–60.49). Importantly, baseline NT-proBNP levels did not significantly predict an unfavorable outcome after 26-weeks of empagliflozin treatment.

**Conclusion** Baseline levels of SIRT2, SIRT4, miR-182-5p, and miR-302a-3p were identified as predictors of ΔLVEF < 11% changes after 26-weeks of treatment, which suggests their potential for stratifying responders and non-responders to empagliflozin. The combined panel of these markers demonstrated the highest predictive accuracy, suggesting

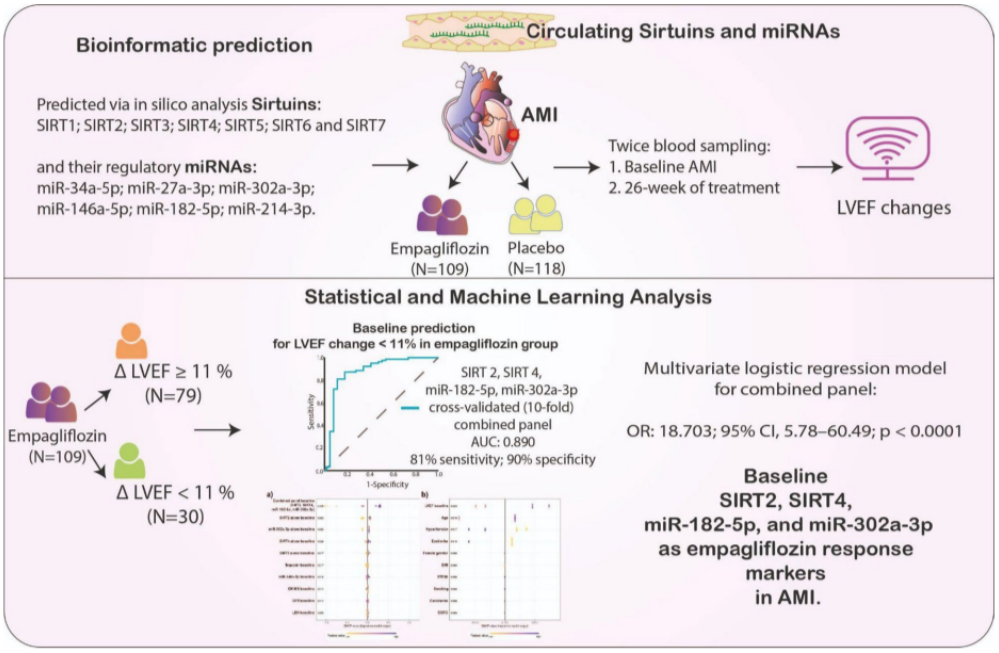
*Correspondence:
Marek Postula
marek.postula@wum.edu.pl

Full list of author information is available at the end of the article

the epigenetic influence of SGLT2 inhibitors and the potential for genomic characterization in personalized treatment approaches.

**Keywords** Empagliflozin, SGLT2 inhibitors, Acute myocardial infarction, Sirtuin, Pharmacogenomics

**Graphical abstract**




**Research Insights:**

What is currently known about this topic?

1. SGLT2 inhibitors are primarily used to manage type 2 diabetes and have shown cardioprotective effects.
2. SGLT2 inhibitors improve myocardial metabolism and reduce inflammation and oxidative stress in AMI patients.
3. Current therapies lack specific molecular targets for optimal cardioprotection and patient-specific treatment strategies.

What is the key research question?

- What are the specific effects of empagliflozin on sirtuin and miRNA expressions in AMI patients, and how can these molecular changes inform the development of targeted therapies?

What is new?

1. Identification of specific miRNAs and sirtuins significantly modulated by empagliflozin treatment.
2. Establishing a link between changes in miRNA/sirtuin profiles and patient responses to empagliflozin.
3. Demonstration of potential new biomarkers (SIRT2, SIRT4, miR-182-5p, miR-302a-3p) for predicting empagliflozin's therapeutic outcomes in AMI.

How might this study influence clinical practice?

- This study highlights potential biomarkers for personalizing treatment in AMI, which could lead to more targeted, effective therapeutic strategies and improved patient outcomes in cardiovascular care.

## Introduction

Sodium-glucose cotransporter-2 (SGLT2) inhibitors are an increasingly applied group of drugs in treatment of type 2 diabetes [1]. In addition to their antidiabetic action, studies have shown their pleiotropic effect that

plays a potential role in the treatment of cardiovascular disease (CVD), i.e. heart failure (HF) or chronic kidney disease (CKD). Cardiovascular benefits of SGLT2 inhibition include improving cardiac energy metabolism, anti-inflammatory effect, prevention of ischemia/reperfusion injury, enhancement of autophagy and lysosomal degradation, and reduction of oxidative stress [2].

The growing interest of application SGLT2 inhibitors treatment in HF, and CVDs, including acute myocardial infarction (AMI), is currently observed. AMI remains the most common cause of ischemic HF, despite the advances in the treatment of coronary artery disease [3]. Within 30 min of ischaemia, cardiomyocyte structural alterations and oedema develop, leading to progressive apoptosis. Acute contractile dysfunction occurs due to oxidative stress and calcium overload. Reperfusion causes a second wave of injury by the production of reactive oxygen species (ROS). Beside the successful epicardial reperfusion, embolization of thrombotic debris, plugging by inflammatory cells and secretion of vasoactive regulator from damaged endothelium causes to ongoing microvascular diseases in up to 50% of patients [4]. Myocardial injury promotes to activation of the inflammatory cascade, consisting of early neutrophil ingress followed by monocyte-macrophage infiltration. Between days 3–5 following AMI, there is a transition from inflammation to repair, with activation of fibroblasts resulting in fibrosis [5]. The precise contribution of the different pathophysiological components (e.g., fibrosis, inflammation, oxidative stress) to ischemic injury can be due to heterogeneity, and understanding mechanistic pathways will be key to identifying novel therapeutic strategies. In this context, it should be noted that recent experimental studies showed multiple benefits from SGLT2 inhibition in the animal model of AMI [6, 7]. Potential mechanisms of action focus on the inhibition of various pathological processes including cardiomyocyte necrosis, neurohormonal activation, and reperfusion injury [8–11]. SGLT2 inhibition may also lead to improvement in outcomes by augmenting endothelial function and vasodilatation [12], myocardial energy metabolism [6, 9, 13], and preservation of cardiac contractility while attenuating pathways of oxidative stress to improve coronary blood flow and ventricular unloading [7, 9, 14–17]. However, there is a lack of human studies evaluating molecular mechanisms of action and clinical effects of SGLT2 inhibitors in the treatment regimen after AMI. Nevertheless, in the randomized EMPACT-MI trial, initiating empagliflozin soon after AMI did not significantly reduce the primary composite of all-cause death or first heart-failure hospitalization versus placebo, underscoring the need for further mechanistic and clinical research and to access which group of patients would benefit from SGLT2 inhibitor therapy after AMI [18].

One interesting hypothesis is that activated sirtuin pathways may be a major protective factor in patients after AMI [19]. Sirtuins are a family of redox-sensitive nicotinamide adenine dinucleotide-dependent deacetylases and are activated by nutrient deprivation and mediate the ability of caloric restriction to preserve organ function and prolong organismal survival [20]. According to previous research, there is an inverse relationship between the activity of SGLT2 and the activity of sirtuins [21]. SGLT2 acts as a central sensor for the nutrient homeostasis of the organism, and pharmacologic inhibition of SGLT2 would be expected to cause an upregulation of sirtuins. In fact, SGLT2 inhibitors enhance the activity of sirtuin 1 (SIRT1, first-generation member of the sirtuin family) and its downstream effectors, even in organs that do not express SGLT2 [22–24]. The activation of SIRT1 and its ability to reduce oxidative stress and inflammation and ameliorate fibrosis may be the major mechanism by which SGLT2 inhibitors exert cardioprotective effects after AMI against HF development, both experimentally and clinically [25].

Epigenetic modifications due to therapy with SGLT2 inhibitors, including altered expression of non-coding RNAs (ncRNAs), including microRNAs (miRNAs), can be identified as a factor affecting sirtuin pathway activity and be a link between SGLT2 inhibitors and sirtuins. The ncRNAs are regulatory RNAs that could modulate different steps in the transcription and translation processes [26]. The ncRNAs are powerful, flexible, and pervasive cellular regulators. They are among the most critical molecules that many drugs, including SGLT2 inhibitors, can affect and subsequently cause changes in the cellular signalling pathways [27]. To date, several studies suggest that the ncRNAs associated with sirtuin pathways are involved in the progression of CVDs by regulating pathogenicity-related gene expression [28]. A novel understanding of the ncRNA language and their role in signalling pathways after AMI and their modification under therapy could be assessed to understand the mechanisms underlying the beneficial effects of SGLT2 inhibitors.

In our study, we aim to thoroughly assess the molecular effects of SGLT2 inhibitors, particularly empagliflozin, and its pharmacogenomic impact on ncRNA expression associated with Sirtuin pathways. We performed bioinformatic analysis to select the top regulated ncRNA targets for Sirtuins further investigation and to validate their expression levels in patient's plasma post-AMI. Lastly, we analyzed to assess the utility of in silico-predicted ncRNAs and sirtuins as biomarkers for outcome prediction for stratification of drug-responders in AMI. Thus, we evaluated the effect of sirtuin pathway modifications in patients after AMI receiving empagliflozin.

## Methods

### Study group

The study was approved by the relevant regulatory authorities, by the Ethics Committee of the Medical University of Graz, Austria (EK 29–179 ex 16/17; EudraCT 2016–004591-22) and registered on ClinicalTrials.gov (NCT03087773). EMMY clinical trial samples were used for the current biomarker substudy and EMMY was conducted in full conformity with the 1964 Declaration of Helsinki and all subsequent revisions, as well as in accordance with the guidelines laid down by the International Conference on Harmonization for Good Clinical Practice (ICH GCP E6 guidelines). EMMY was managed and led by the Interdisciplinary Metabolic Medicine Trials Unit at the Medical University of Graz, Austria. The detailed inclusion and exclusion criteria and demographic table for the whole cohort were previously published [29].

Out of 476 patients that were included in the EMMY trial, 299 patients paired plasma samples with required clinical data were available for this analysis, 72 small/total RNA did not pass the quality control. Therefore, in this current analysis, a total of 227 patients were included with baseline, and 26-weeks after treatment, 454 longitudinal samples were analyzed (Fig. 1). Medical records were obtained, and demographic, clinical, and laboratory data of patients receiving empagliflozin are presented in Table 1. EDTA blood plasma was used for RNA analysis and plasma was kept in -80 ˚C until the day of experiments in the Medical University of Warsaw. No freeze–thaw cycles were performed during the experiments.

For further analysis, we divided the patients based on the change in LVEF (ΔLVEF) between baseline and 26 weeks, using a cut-off of 11%. This threshold was determined from the third quartile distribution in the empagliflozin group, where the median ΔLVEF was 6% (0% at the first quartile and 11% at the third quartile). Consequently, 27% of patients in the empagliflozin group had a ΔLVEF ≥ 11% after 26 weeks of treatment. Additionally, the selection of this cut-off value can be further substantiated by Strange et al. study, where the decrease of 10-units or more in ΔLVEF was associated with two fold higher mortality [30]. Out of 227 patients, 171 (75.33%) had the ΔLVEF lower than 11% (after 26-weeks of treatment), regardless of randomization to empagliflozin or placebo treatment.

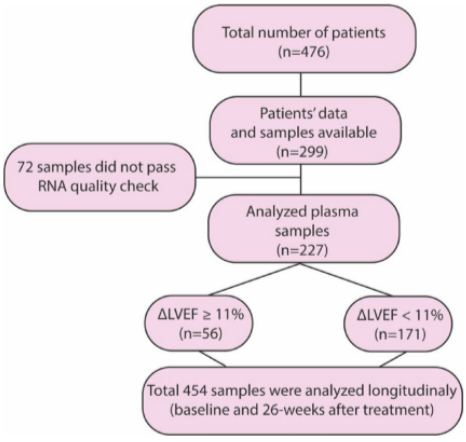



**Fig. 1** Sample workflow

### Bioinformatic analysis

***Generation of SGLT2 interaction network:*** The SGLT2 interaction network was constructed and visualized as described before by us [31] using the first-level SGLT2 interactors (direct interactors) and both level interactors obtained from the complete Human interactome version 11.0b (from 17 October 2020 to 12 August 2021) using stringApp v1.5.1 through Cytoscape software v3.8.2 [32, 33]. The network was further expanded by retrieving second-level SGLT2 interactors (indirect interactors) from the human interactome by mapping all neighbor nodes of the first-level SGLT2 interactors.

***Selection of Key Term-Related Gene Lists:*** Gene lists of interests were obtained as follows: first-level SGLT2 interactors, both-levels SGLT2 interactors obtained from SGLT2 interaction network. Top 100 SIRT1-7 interactors were obtained using String database. Genes associated with inflammation, fibrosis, oxidative stress and hypoxia were obtained from Gene Ontology database through the biomaRt R package [34]. Genes associated with AMI and HF were retrieved from Disgenet database [35].

***miRNA predictions:*** Tissue-specific expression of human miRNAs in blood and plasma was retrieved from the TISSUES 2.0 database using download "all channels Integrated" for human organism [36, 37]. Gene-tissue associations were obtained from the Jensen TISSUES database, which integrates evidence from transcriptomics (Exon Array, GNF, Human Protein Atlas RNA-seq, and RNA-seq Atlas), proteomics, and text mining. For each gene-tissue pair, these sources are combined into a unified confidence score (0–1), reflecting the strength and consistency of the evidence [36]. A threshold of ≥ 0.5 was applied to retain associations with at least medium reliability. We selected 1676 pre-miRNAs with expression confidence at least 0.5 from 1 in blood plasma and blood serum. Further we performed miRNAs prediction using as targets all genes from our lists of interest. For miRNA predictions we used multimiR package version 1.22.0, we looked for top 20% of predictions in 14 miRNA databases within the provided list of targets of interest [38].

***Targets predictions:*** We conducted target-miRNA predictions using multimiR R package for all genes regulated by the top miRNAs, including those not present on

**Table 1** Baseline demographic data for empagliflozin randomized patients

| Characteristic | Overall of empagliflozin-takers (n = 109) | ΔLVEF ≥ 11% changes after 26-weeks of empagliflozin treatment (n = 30; 28%) | ΔLVEF < 11% changes after 26-weeks of empagliflozin treatment (n = 79; 72%) | Complete data | *p* value |
|---|---|---|---|---|---|
| LVEF change | 6 (0.5–11.0) | 15 (11–20) | 2 (-3–7) | 100% | **< 0.001** |
| Age (years) | 58 (52–64) | 57 (49–61) | 58 (53–64) | 100% | 0.086 |
| Female sex. n (%) | 22 (20) | 9 (30) | 13 (17) | 100% | 0.116 |
| Body mass index (kg/m2) | 28 (25–30) | 29 (25–30) | 28 (25–30) | 100% | 0.846 |
| Type 2 diabetes. n (%) | 10 (9) | 1 (3) | 9 (11) | 100% | 0.193 |
| Hypertension. n (%) | 41 (38) | 16 (53) | 25 (32) | 100% | **0.037** |
| Dyslipidaemia. n (%) | 33 (30) | 12 (40) | 21 (27) | 100% | 0.173 |
| Smoking (active or former). n (%) | 75 (69) | 22 (73) | 53 (67) | 100% | 0.530 |
| Coronary artery disease. n (%) | 14 (13) | 4 (13) | 10 (13) | 100% | 0.925 |
| History of stroke. n (%) | 2 (2) | 0 (0) | 2 (3) | 100% | 0.379 |
| History of myocardial infarction. n (%) | 6 (6) | 1 (3) | 5 (6) | 100% | 0.540 |
| Depression. n (%) | 9 (8) | 4 (13) | 5 (6) | 100% | 0.235 |
| Coronary angiography vessel status | | | | | |
| 3-vessel disease. n (%) | 28 (26) | 6 (20) | 22 (28) | 100% | 0.402 |
| 2-vessel disease. n (%) | 40 (37) | 11 (37) | 29 (37) | 100% | 0.997 |
| 1-vessel disease. n (%) | 41 (38) | 13 (43) | 28 (35) | 100% | 0.448 |
| Treatment | | | | | |
| ACE-I/ARB. n (%) | 105 (96) | 29 (97) | 76 (96) | 99% | 0.828 |
| ARNI. n (%) | 1 (1) | 0 (0) | 1 (1) | 100% | 0.536 |
| Beta-blocker. n (%) | 103 (95) | 29 (97) | 74 (94) | 100% | 0.540 |
| MRA. n (%) | 43 (39) | 10 (33) | 33 (42) | 100% | 0.421 |
| Loop diuretic. n (%) | 14 (13) | 3 (10) | 11 (14) | 100% | 0.584 |
| Statin. n (%) | 108 (99) | 30 (100) | 78 (99) | 100% | 0.536 |
| Ezetimibe. n (%) | 16 (15) | 9 (30) | 7 (9) | 100% | **0.005** |
| Calcium channel blocker. n (%) | 7 (6) | 3 (10) | 4 (5) | 100% | 0.348 |
| Platelet lowering drugs. n (%) | 109 (100) | 30 (100) | 79 (100) | 100% | N/A |
| Anticoagulation drugs. n (%) | 7 (6) | 0 (0) | 7 (9) | 100% | 0.092 |
| Metformin. n (%) | 7 (6) | 1 (3) | 6 (8) | 100% | 0.418 |
| DPP4 inhibitor. n (%) | 2 (2) | 0 (0) | 2 (3) | 100% | 0.379 |
| Sulfonylurea. n (%) | 1 (1) | 0 (0) | 1 (1) | 100% | 0.536 |
| GLP1-RA. n (%) | 0 (0) | 0 (0) | 0 (0) | 100% | N/A |
| Insulin. n (%) | 2 (2) | 0 (0) | 2 (3) | 100% | 0.379 |
| Laboratory parameters | | | | | |
| NT-proBNP (pg/mL) | 1443 (883–2685) | 1323 (839–2855) | 1506 (947–2685) | 99% | 0.845 |
| eGFR (mL/min/1.73 m2) | 90.0 ± 16.1 | 93.5 ± 19.4 | 88.6 ± 14.5 | 100% | 0.155 |
| HbA1c (%) | 5.6 (5.4–5.9) | 5.6 (5.4–5.7) | 5.6 (5.4–6) | 96% | 0.359 |
| Kreatinin (mg/dl) | 0.9 ± 0.2 | 0.9 ± 0.3 | 0.9 ± 0.2 | 100% | 0.795 |
| Troponin T (ng/L) | 3531 (1956–5911) | 3057 (2107–5518) | 3597 (1813–6725) | 99% | 0.930 |
| Total cholesterol (mg/dL) | 198.3 ± 41.2 | 201.7 ± 35.6 | 196.9 ± 43.3 | 99% | 0.592 |
| LDL-cholesterol. (mg/dL) | 126.3 ± 37.1 | 130.0 ± 33.1 | 124.8 ± 38.7 | 97% | 0.514 |
| HDL-cholesterol (mg/dL) | 44.9 ± 11.8 | 44.6 ± 11.2 | 45.0 ± 12.1 | 98% | 0.897 |
| Aspartate aminotransferase (IU/L) | 204 (141–321) | 173 (129–234) | 218 (147–386) | 98% | 0.060 |
| Alanine aminotransferase (IU/L) | 55 (37–77) | 51 (28–70) | 56 (42–82) | 98% | 0.050 |
| Gamma-glutamyltransferase (IU/L) | 30 (21–50) | 34 (22–49) | 27 (20–59) | 97% | 0.961 |
| Ferritin | 196 (118–304) | 196 (110–295) | 199 (121–304) | 98% | 0.627 |
| Studied biomarkers | | | | | |
| SIRT1* | 9.57 (8.92–10.70) | 9.36 (8.87–10.10) | 9.76 (8.97–11.21) | 83% | 0.989 |
| SIRT2* | 10.19 (8.89–11.68) | 8.80 (8.69–9.03) | 11.52 (10.19–11.80) | 75% | **< 0.0001** |
| SIRT3* | 10.62 (9.51–11.32) | 9.60 (8.88–11.14) | 10.84 (9.86–11.41) | 66% | 0.391 |
| SIRT4* | 10.38 (8.59–11.42) | 8.51 (8.44–9.55) | 10.92 (10.18–11.65) | 73% | **0.0001** |
| SIRT5* | 10.11 (8.89–11.09) | 9.27 (8.63–10.81) | 10.86 (9.63–11.16) | 73% | 0.166 |

**Table 1** (continued)

| Characteristic | Overall of empagliflozin-takers (n = 109) | ΔLVEF ≥ 11% changes after 26-weeks of empagliflozin treatment (n = 30; 28%) | ΔLVEF < 11% changes after 26-weeks of empagliflozin treatment (n = 79; 72%) | Complete data | *p* value |
|---|---|---|---|---|---|
| SIRT6* | 10.88 (9.69–11.71) | 10.31 (9.27–11.13) | 10.94 (9.79–11.30) | 65% | 0.726 |
| SIRT7* | 11.09 (9.91–11.72) | 8.84 (8.74–11.31) | 11.46 (10.31–11.79) | 66% | 0.438 |
| miR-34a-5p* | 10.58 (9.49–11.75) | 10.67 (9.49–11.50) | 10.56 (9.44–11.84) | 57% | 0.631 |
| miR-27a-3p* | 12.43 (11.58–13.59) | 12.39 (11.80–12.84) | 12.44 (11.46–13.76) | 96% | 0.401 |
| miR-302a-3p* | 12.20 (9.42–15.14) | 10.24 (8.84–13.06) | 12.91 (10.40–15.67) | 88% | **0.004** |
| miR-146-5p* | 12.65 (11.82–13.46) | 12.71 (10.32- 13.41) | 12.65 (11.96–13.63) | 86% | 0.151 |
| miR-182-5p* | 11.04 (9.52–13.53) | 9.87 (9.30–11.03) | 12.36 (10.21–14.16) | 83% | **0.002** |
| miR-214-5p* | 10.39 (9.60–11.02) | 10.24 (9.64–10.53) | 10.57 (9.27–11.33) | 87% | 0.100 |

Non-normally distributed data are presented as median with interquartile range, whereas normally distributed data are presented as mean with standard deviation. *P* values were calculated using the Chi-square test for categorical variables, and either the Mann–Whitney U test (for non-normally distributed data) or Student's t-test (for normally distributed data). Abbreviations used include: ACE-I. angiotensin-converting-enzyme inhibitors; ARB. Angiotensin receptor blocker; eGFR. estimated glomerular filtration rate; GLP1-RA. glucagon-like peptide-1 receptor agonist; HbA1c. Glycated hemoglobin; IQR. interquartile range; miRNA. Micro-RNA; MRA. Mineralocorticoid receptor antagonist; NT-proBNP. N-terminal fragment of pro-brain natriuretic; SIRT. Sirtuin; SD for standard deviation. *All miRNA and gene expression results are shown as LOG10

our gene lists of interest. We focused on genes regulated by the highest number of these top miRNAs, particularly those interacting with any of the sirtuins, present within the SGLT2 interaction network, and associated with the ontological terms relevant to our research.

***Ranking of miRNAs and targets:*** To aggregate data and evaluate how many targets from each gene list of interest were regulated by the analysed miRNAs we used, developed by us wizbionet R package [39]. Aggregation and summarizing of the rows across multiple columns was performed function using the col_agrecounter() function. Clusterization of gene lists was performed using the clusterizer_oneR() function based on the machine learning (ML) algorithm OneR [40]. This function enables the comparison of data sets of different lengths that have associated numeric values. Prioritization of the analyzed gene lists was based on the scores assigned after data aggregation and counting. It helped to avoid arbitrary selection of top candidates, by dividing the analyzed data set into four clusters using the OneR package. If clusters were too small, it applied k-means clustering. As top miRNAs/genes, we recognized entities present in the first two top clusters (cl1, cl2).

***Construction of SIRT-miRNA-target interaction network:*** Sirtuin-related interaction network was constructed in Cytoscape software based on the miRNA target interactions data provided by the multimiR package. We visualised interactions between top miRNAs, seven sirtuins and top targets associated with SGLT2 interaction network using Cytoscape software. On the nodes, we visually mapped associations with our gene lists of interest. Nodes were organized using a tree layout (Fig. 2).

#### RNA analysis

The Maxwell® RSC 48 automatic system was used for total RNA extraction using 400 µl of plasma aliquots. Maxwell RSC miRNA Plasma and Serum isolation kits (REF AS1680) were used for total RNA extraction as the manufacturer's recommendations. Plasma was preprocessed with Proteinase K and Lysis Buffer in the volumes described within the manufacturers' protocols. The mixture will be placed on a vortex mixer at 3,000 rpm for 5 s, and then left at 37 °C for 15 min. After transferring prepared lysate to the Cartridge DNase I Solution was added. Total RNA was eluted by 50 µl of nuclease-free water (Applied Biosystems, CA). The RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE) and Qubit RNA high sensitivity (Invitrogen). The extracted RNA yield and quality was evaluated using 2100 Bioanalyzer (Agilent Genomics, USA) as well as by fluorometric assay. Afterwards, miRNA expressions were detected by quantitative polymerase chain reaction (qPCR) using TaqMan miRNA Assay kits (catalog number A25576, assay ID: hsa-miR-302a-3p; hsa-miR-27a-3p; hsa-miR-34a-5p; hsa-miR-146a-5p; hsa-miR-182-5p; hsa-miR-214-3p), moreover, sirtuin gene expression using TaqMan Gene expression Assays (catalog number: 4331182, assay ID: Hs01009006_m1, Hs01560289_m1, Hs00953477_m1, Hs01015516_g1, Hs00978331_m, Hs07287877_m1, Hs01034735_m1) by using CFX384 Touch Real-Time PCR Detection System (BioRad Inc. Hercules, California, USA). Cel-miR-39 was added as a spike-in control during the miRNA extraction phase and served as an exogenous normalization control. Additionally, all samples were normalized to the synthetic spike-in cel-miR-39, which was added in equal amounts during RNA isolation. In addition, one randomly selected sample was used as an internal reference across all qPCR runs [41, 42]. All reactions were performed in triplicates, and mean values were used in statistical analysis as described before [43,

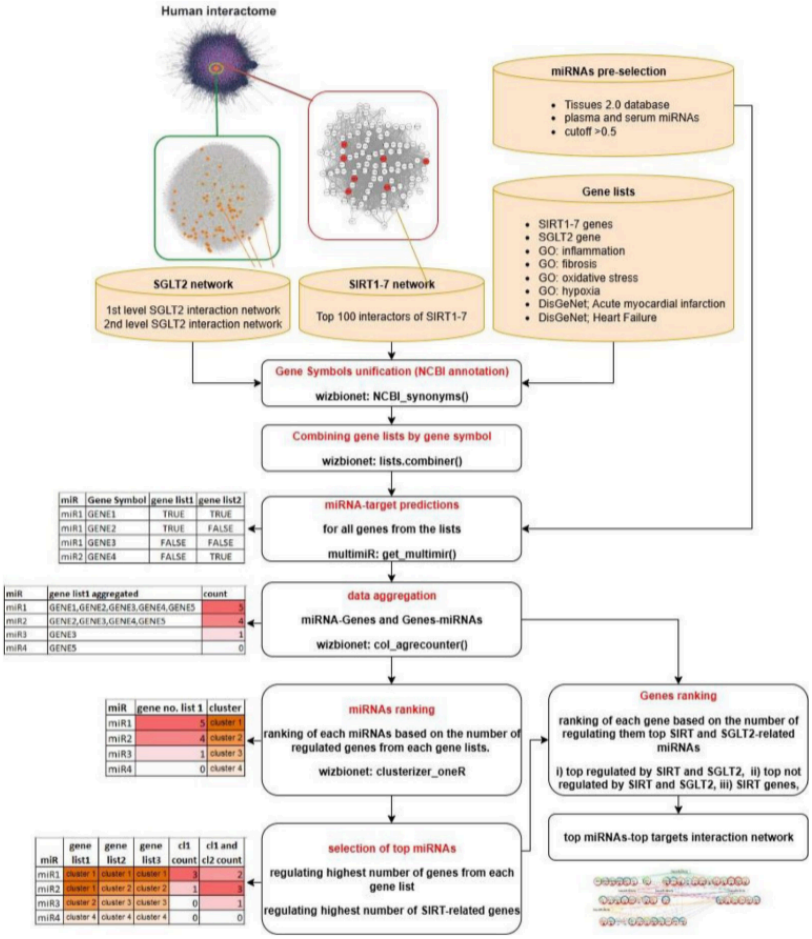



**Fig. 2** Bioinformatic workflow of selection of the top miRNAs and top targets in context of sirtuins and SGLT2 related activity

44]. MiRNA expressions were expressed as 2-ΔΔCT [45], results were log-10 transformed for statistical analysis.

### SHAP analysis

This study used laboratory and demographic predictors extracted from a clinical dataset. Relevant variables were identified separately for the laboratory and demographic domains and coerced to numeric to form model inputs. Missing values were handled with a custom preprocessing function (NoNA.df) from the wizbionet R package, which standardized and removed nonstandard NA encodings to yield a complete input matrix. For the laboratory block, features were selected by matching the header for the keyword "laboratory"; deltaLVEF was excluded. The outcome was binarized (0 = good, 1 = bad).

Models were trained with gradient-boosted trees (XGBoost, binary:logistic) on a stratified 70/30 train-test split with early_stopping_rounds = 10, and selected by maximizing validation AUC with an overfitting guard (training AUC < 0.99). For the laboratory feature set (56 predictors), the best configuration was $\eta = 0.01$, max_depth = 3, $\gamma = 3$, subsample = 0.8, colsample_bytree = 0.8 (others default), which early-stopped at 37 trees (best_ntreelimit = 37) with validation AUC = 0.8313 and training AUC = 0.9736; this model was used for SHAP on the held-out test set. For the demography feature set (37 predictors), the best configuration was $\eta = 0.03$, max_depth = 4, $\gamma = 3$, subsample = 0.8, colsample_bytree = 0.8, which early-stopped at 4 trees (best_ntreelimit = 4) with validation AUC = 0.7375 (training AUC = 0.8799). These

configurations were fixed for interpretation. SHAP values were computed on the independent test set using TreeSHAP (via SHAPforxgboost), which decomposes each prediction on the margin (log-odds) scale into a bias term plus per-feature contributions that sum to the model output, $f(x_i) = \phi_{0o} + \Sigma_j \phi_{ij}$. Positive SHAP values increase the log-odds of the "bad" class, whereas negative values decrease it. Global importance was summarized as the mean absolute SHAP value across test samples, and distributions/directionality were visualized with a SHAP summary (beeswarm) plot. The workflow was automated with custom R functions to support reproducibility. R packages which were used: xgboost (training/prediction), SHAPforxgboost (SHAP; with ppcor and WGCNA in sensitivity analyses), ggplot2 (graphics), dplyr and wizbionet (data handling), and additionally Hmisc, cluster, caret, and pROC.

### Statistical analysis

Categorical variables were presented as a number and percentage, continuous variables were expressed as median and interquartile range (IQR). Two-categorical variables statistics were analyzed by the Chi-Square test. The normality of distribution is evaluated by Shapiro–Wilk test. Depending on the normality of the distribution, the Student's t-test or Mann–Whitney test was used for unpaired samples, and for paired data, the Wilcoxon test was used. To assess the predictive value of baseline SIRT2 and SIRT4, miR-182-5p, miR-302a-3p for ΔLVEF < 11% (using an 11% cut-off based on the 3th quartile), we used receiver operating characteristic (ROC) analysis. To enhance the robustness of the statistical analysis given the limited sample size, bootstrapping with tenfold cross-validation was performed for ROC curve analysis. The Hosmer–Lemeshow test was applied to assess the goodness of fit and calibration of the logistic regression models. No correction for multiple comparisons was applied to Mann–Whitney U tests when a single variable was compared between two groups. Missing data were addressed using median imputation to preserve the integrity and reliability of the biomarker panel analysis. Baseline high SIRT2, high SIRT4, high miR-182-5p, and high miR-302a-3p expression as a panel, age (years), female sex, hypertension, BMI, ezetimibe were included in the multivariate logistic regression analysis model. All tests were two-sided with a significance level of $p < 0.05$. Calculations were performed using SPSS version 22.0 (IBM Corporation, Chicago, USA). Graphs were improved by Adobe Illustrator 24.0.2.

## Results

### Participants

Although our study included 227 patients in total (both empagliflozin and placebo), the longitudinal analysis comprised 454 samples. Importantly, for the drug-response analysis we focused exclusively on the empagliflozin-treated patients, i.e., 109 individuals with paired measurements before and after treatment ($109 \times 2 = 218$ samples). Patients were stratified according to the ΔLVEF between baseline and 26 weeks. A cut-off value of 11% was applied, corresponding to the third quartile of the ΔLVEF distribution within the empagliflozin treatment group. Therefore, the cut-off was derived solely from the treatment arm and applied consistently within that context, not across both arms and only empagliflozin randomized patients' characteristics were presented in Table 1 based on ΔLVEF < 11% outcome. The demographic table shows that empagliflozin-takers based on ΔLVEF ≥ 11% vs < 11% subgroups were clinically matched for the SIRTs and miRNAs expression analysis on baseline, including age, sex, and body mass index (BMI) ($p = 0.086$; $p = 0.116$; $p = 0.846$, respectively). On the other hand, patients taking empagliflozin with a ΔLVEF ≥ 11% more frequently had hypertension and were more often treated with ezetimibe compared to those with a ΔLVEF < 11% ($p = 0.037$ and $p = 0.005$, respectively). Besides, whole cohort (placebo and empagliflozin groups) demographics based on ΔLVEF < 11% outcome were also presented in Supplemental Table 1.

### Bioinformatic analysis results

**In silico** ***prediction analysis by computational approach:*** To prepare the ground for interpretation of the transcriptomic results and their integration with the clinical data, we performed preliminary bioinformatic analysis. In order to obtain the context of SIRTs and SGLT2 spectrum of interactions, we recreated the SGLT2 interaction network, by retrieving all first-level SGLT2 interactors from the Human interactome String database. Further, we expanded it by including second-level interactors through mapping all the neighbor nodes of the first-level SGLT2 interactors as described before [31]. The SGLT2 network consisted of 5225 nodes, including SGLT2 and its 65 first-level interactors and 5160 s-level interactors.

Next we generated another network using human interactome for SIRT-1–7 where we extracted the top 100 of their interactors. Further, for both networks we performed visual mapping of gene annotations related to key terms (oxidative stress; inflammation; fibrosis; hypoxia–ischemia). Summarizing we obtained following gene lists: gene list with seven sirtuins, gene list with SGLT2 synonyms, first-level SGLT2 interactors, both-levels SGLT2 interactors, top 100 SIRT1-7 interactors, inflammation, fibrosis, oxidative stress, hypoxia, AMI and HF.

***miRNA prediction and ranking based on SIRTs and SGLT2 related gene lists:*** Further, we performed miRNA prediction, using multimiR package, based on miRNA

ability to regulate above-mentioned targets. We focused on miRNAs expressed only in human blood or plasma according to the Tissues 2.0 database. In total, we identified 1234 miRNAs which were able to regulate any of the analysed targets. MiRNA-target predictions were combined with analysed genes, aggregated, divided by four clusters based on the number of targets regulated and ranked.

We divided miRNAs into two groups. In the first miRNAs regulated SGLT2 and at least one sirtuin from seven. In the second miRNAs regulated top two clusters for the following gene lists: seven sirtuins, first-level SGLT2 interactors, and both-level SGLT2 interactors. Further miRNAs were sorted based on presence in the first group, second miR group, number of first clusters regulated and then number of first and second OneR clusters regulated. Summarizing, we obtained 6 top miRNAs targeting the highest number of SIRTs and components of our gene lists of interest. Best in the ranking were hsa-miR-34a-5p, hsa-miR-27a-3p and hsa-miR-302a-3p (Table 2).

***miRNAs target ranking based on SIRTs and SGLT2 related gene lists*:** In this step, we performed identification of the top genes potentially playing a role in Sirtuins regulation based on identification of top miRNAs involved in SIRT-SGLT2 regulation. For this part of the study, we performed miRNA-target predictions for all genes regulated by the top miRNAs, not only present on our lists of interest. We focused on the genes regulated by the highest number of top miRNAs, which were also interacting with any of the sirtuins, present within the SGLT2 interaction network, and associated with ontological terms of our interest. List of top genes is shown in Table 3**.** For further visualisation, we focused on the ones regulated by the highest number of top miRNAs, which were as well interacting with any of the sirtuins, were present within the SGLT2 interaction network and were associated with ontological terms of our interest (Fig. 3 ).

Taking into account the role of Sirtuins as transcription regulators, we decided to screen ENCODE Transcription Factor Binding Site Profiles in order to see if the SGLT2 (SLC5A2) promoter region could be their target. We identified such a binding for the SIRT6_K562_hg19_1 [46]. SIRT6 also targeted other components of the SGLT2 network including SIRT1 (Fig. 3).

### Sirtuins expression alterations based on treatment and time changes

Assessment of changes in Sirtuin mRNAs expression levels is depicted in Fig. 4**.** It illustrates the relative expression of circulating mRNAs of Sirtuins in patients with AMI taking placebo or empagliflozin, at two different time intervals: upon admission and 26-week after treatment. SIRT4 was significantly decreased in the empagliflozin group compared to placebo after 26-weeks of treatment ($p = 0.018$). On the other hand, SIRT6 was significantly increased after 26-week in the empagliflozin group compared to placebo ($p < 0.001$). Moreover, we observed significant down-regulation in SIRT6 after the treatment of placebo ($p = 0.006$).

### High Baseline expression of SIRT2 and SIRT4 are associated with ΔLVEF < 11% after 26-weeks of empagliflozin treatment following AMI

Patients with ΔLVEF < 11% had significantly higher expression levels of both SIRT2 and 4 when compared to those with ΔLVEF ≥ 11% in empagliflozin group ($p < 0.001$, $p = 0.001$, respectively) **(**Fig. 5A and 5C**).** According to the ROC curve analysis, a high baseline SIRT2 expression presents predictive utility in assessment of the ΔLVEF < 11% in empagliflozin group (AUC: 0.806, $p < 0.001$) **(**Fig. 5B**).** Similar results were found also for SIRT4 (AUC: 0.765, $p < 0.001$) (Fig. 5 D). We did not observe any significant differences in the levels of SIRT 1, 3, 5, 6, and 7 between the groups treated with empagliflozin (Table 1).

### MiRNA expression alterations based on treatment and time changes

Assessment of alterations in miRNA expression levels is presented in Fig. 6. It illustrates the relative expression

**Table 2** Top miRNAs targeting the highest number of SIRT1-7 genes, SGLT2 and first-level SGLT2 interactors

| mature mirna id | # of SIRT genes (cluster 1 / cluster 2 / cluster 3 / cluster 4) | Targets SGLT2 gene | # of SIRT genes | # of 1st level SGLT2 interactors | # of 1st and 2nd level SGLT2 interactors | # of SIRT interactors | # of inflammation genes | # of fibrosis genes | # of oxidative stress genes | # of hypoxia genes | # of MI | # of HF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **hsa-miR-34a-5p** | SIRT1\|SIRT2\|SIRT5\|SIRT6\|SIRT7 | yes | 5 | 16 | 1892 | 60 | 224 | 133 | 54 | 163 | 661 | 559 |
| **hsa-miR-27a-3p** | SIRT1\|SIRT3\|SIRT4\|SIRT6 | yes | 4 | 22 | 2157 | 38 | 262 | 130 | 56 | 149 | 725 | 601 |
| **hsa-miR-302a-3p** | SIRT1\|SIRT2\|SIRT3\|SIRT5 | yes | 4 | 14 | 1126 | 24 | 139 | 77 | 26 | 95 | 385 | 334 |
| hsa-miR-146a-5p | SIRT3 | yes | 1 | 22 | 1643 | 33 | 188 | 101 | 43 | 123 | 544 | 456 |
| hsa-miR-182-5p | SIRT1 | yes | 1 | 12 | 1633 | 39 | 187 | 117 | 43 | 126 | 518 | 431 |
| hsa-miR-214-3p | SIRT1 | yes | 1 | 8 | 1118 | 33 | 128 | 75 | 30 | 103 | 404 | 332 |

**Table 3** List of top genes regulated by top miRNAs. Genes marked with green showed the strongest regulation by top miRNAs, are SIRT1 interactors, are involved in the SGLT2 interaction network and are associated with multiple GO terms. Genes marked with blue showed associations as above, but they were not present on analysed GO lists/ or the association is still not known. Genes marked with red are SIRTs with complete information regarding regulation by top miRNAs and their association with analyzed GO lists. Top three miRNAs from Table 2 are bolded

| Symbol NCBI | # mature miRNAs regulating SIRT | # mature selected top miR | # GO terms | Selected regulatory miRNAs | SIRT genes | 1st level SGLT2 interactor | 1st and 2nd level SGLT2 interactor | Top 100 SIRTs interactors | inflammation | fibrosis | oxidative stress | hypoxia | MI | HF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PPARA | 33 | 6 | 6 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-939-5p | | | + | + | + | | | + | + | + |
| HIF1A | 24 | 6 | 6 | hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-34a-5p**;hsa-miR-509-5p;hsa-miR-767-3p | | | + | + | + | | | + | + | + |
| CREB1 | 37 | 7 | 5 | hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-767-3p;hsa-miR-939-5p | | | + | + | | + | | + | + | |
| PTEN | 31 | 6 | 5 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-509-5p | | | + | + | | | | + | + | + |
| CLOCK | 37 | 8 | 4 | hsa-miR-146a-5p;hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-509-5p | | | + | + | + | | | | + | |
| FOXO3 | 25 | 6 | 5 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p** | | | + | + | | | | + | + | + |
| IGF1 | 25 | 7 | 5 | hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-509-5p | | | + | + | | + | | | + | + |
| PPARGC1A | 23 | 6 | 5 | hsa-miR-149-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-34a-5p**;hsa-miR-509-5p;hsa-miR-939-5p | | | + | + | | | | + | + | + |
| ATG7 | 22 | 6 | 4 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-509-5p | | | + | + | | | | | + | + |
| MAPK1 | 33 | 9 | 4 | hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-4286;hsa-miR-509-5p;hsa-miR-767-3p | | | + | | | + | | | + | + |
| CDKN1A | 27 | 10 | 4 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-4286;hsa-miR-509-5p;hsa-miR-767-3p;hsa-miR-939-5p | | | + | | | + | | | + | + |
| E2F1 | 25 | 9 | 4 | hsa-miR-182-5p;hsa-miR-214-3p;hsa-miR-296-5p;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-4286;hsa-miR-939-5p | | | + | | | + | | + | + | |
| EGFR | 26 | 6 | 6 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-767-3p | | | + | | + | + | + | | + | + |
| THBS1 LDLR | 25 | 8 | 6 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-509-5p | | | + | | + | + | | + | + | + |
| PDGFRA | 29 | 6 | 4 | hsa-miR-149-5p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-4286;hsa-miR-939-5p | | | + | | + | | | | + | + |
| PIK3CA | 23 | 7 | 4 | hsa-miR-146a-5p;hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p** | | | + | | | + | | | + | + |
| STAT3 | 26 | 7 | 4 | hsa-miR-146a-5p;hsa-miR-182-5p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-509-5p;hsa-miR-767-3p | | | + | | | + | | | + | + |
| VEGFA | 28 | 7 | 4 | hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-767-3p;hsa-miR-939-5p | | | + | | + | | | | + | + |
| ATXN1 | 26 | 6 | 4 | hsa-miR-146a-5p;hsa-miR-214-3p;hsa-miR-296-5p;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-939-5p | | | + | | | | | + | + | + |
| HIPK2 | 36 | 9 | 3 | hsa-miR-149-5p;hsa-miR-182-5p;hsa-**miR-27a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-509-5p;hsa-miR-939-5p | | | + | | | | | | + | + |
| KMT2A | 35 | 9 | 3 | hsa-miR-146a-5p;hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-miR-296-5p;hsa-**miR-34a-5p**;hsa-miR-939-5p | | | + | | | | | | + | + |
| CDK6 | 37 | 11 | 3 | hsa-miR-146a-5p;hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-miR-296-5p;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-767-3p;hsa-miR-939-5p | | | + | | | | | | | |
| CELF1 | 38 | 9 | 2 | hsa-miR-146a-5p;hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-509-5p;hsa-miR-767-3p | | | + | | | + | | | | |
| YWHAZ | 36 | 10 | 2 | hsa-miR-146a-5p;hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-miR-296-5p;hsa-**miR-302a-3p**;hsa-miR-4286;hsa-miR-767-3p;hsa-miR-939-5p | | | + | | | | | | + | |
| VWHAZ | 35 | 9 | 2 | hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p**;hsa-miR-4286;hsa-miR-939-5p | | | + | | | | | | + | |
| SIRT1 | 30 | 8 | 7 | hsa-miR-149-5p;hsa-miR-182-5p;hsa-miR-214-3p;hsa-**miR-27a-3p**;hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-509-5p;hsa-miR-767-3p | + | + | + | | | | + | + | + | + |
| SIRT4 | 8 | 1 | 4 | hsa-**miR-27a-3p** | + | | + | | | | | + | + | |
| SIRT2 | 17 | 4 | 4 | hsa-**miR-302a-3p**;hsa-**miR-34a-5p**;hsa-miR-767-3p;hsa-miR-939-5p | + | | + | | | | | + | + | |
| SIRT3 | 11 | 5 | 4 | hsa-miR-146a-5p;hsa-**miR-27a-3p**;hsa-miR-296-5p;hsa-**miR-302a-3p**;hsa-miR-4286 | + | | + | | | | | | + | + |
| SIRT6 | 16 | 2 | 3 | hsa-**miR-27a-3p**;hsa-**miR-34a-5p** | + | | + | | | + | | | + | + |
| SIRT5 | 24 | 3 | 2 | hsa-**miR-302a-3p**;hsa-miR-30b-3p;hsa-**miR-34a-5p** | + | | + | | | | | | | |
| SIRT7 | 15 | 1 | 2 | hsa-**miR-34a-5p** | + | | + | | | | | | | |

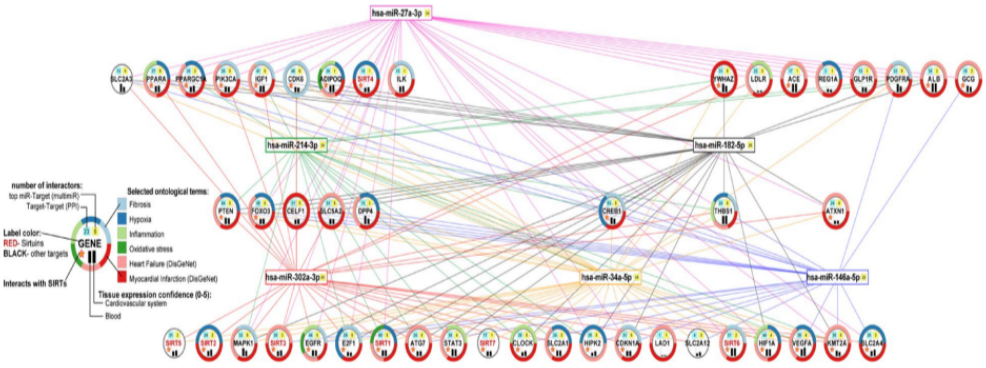



**Fig. 3** Interaction network constructed for the top 6 miRNAs and their targets shown in Table 2 and 3. Star next to the gene indicates the interaction with SIRT. Yellow marked numbers indicate the number of top miRNAs that the gene targets

of circulating miRNAs in patients receiving placebo or empagliflozin, measured at baseline and after 26-weeks of treatment. The miR-34a-5p and miR-214-3p expression significantly increased after the 26-week treatment with empagliflozin ($p = 0.001$, $p = 0.003$ respectively). We observed no significant changes in the expression of miR-27a-3p, miR-302a-3p, miR-146a-5p, and miR-182-5p (data not shown).

### High Baseline expression of miR-182-5p and miR-302a-3p is associated with ΔLVEF < 11% after 26-weeks of empagliflozin treatment post AMI

Patients with ΔLVEF < 11% exhibited significantly higher expression levels of miR-182-5p and miR-302a-3p compared to those with ΔLVEF ≥ 11% ($p = 0.002$ and $p < 0.001$, respectively) at the baseline (Fig. 7A and C). ROC curve analysis revealed that high baseline expression

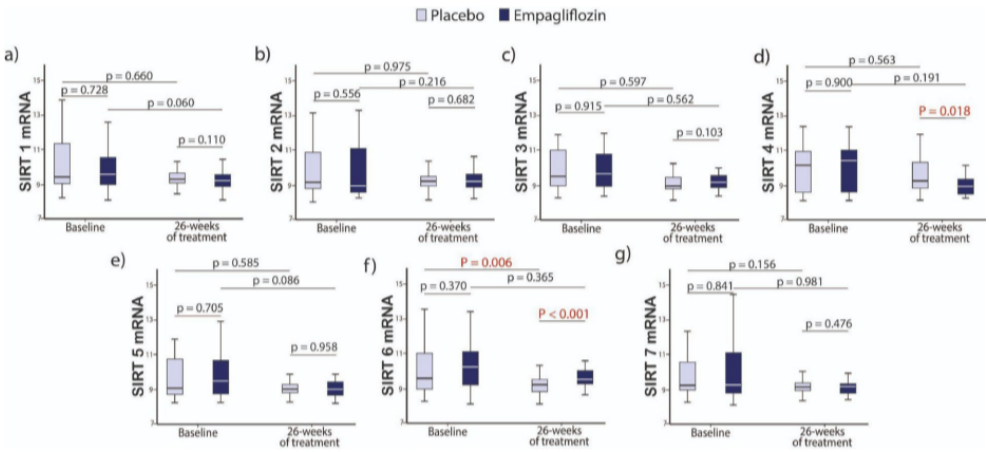



**Fig. 4** Boxplots for SIRT genes expression between the treatments and time proportional changes regarding a) SIRT1; b) SIRT2; c) SIRT3; d) SIRT4; e) SIRT5; f) SIRT6; g) SIRT7. Un-paired data was calculated with Mann Whitney U test, Paired data was calculated by Wilcoxon test. All miRNA expression is presented by LOG10

of miR-182-5p and miR-302a-3p hold predictive value for assessing ΔLVEF < 11% (AUC: 0.716, $p = 0.002$; AUC: 0.757, $p < 0.001$, respectively) (Fig. 7B and D). No significant findings were observed according to other miRNAs whose expression levels were measured in our cohort (miR-27a-3p, miR-146a-5p, miR-182-5p, miR-214-3p) (data not shown).

#### Combined SIRT genes and miRNA expression results as a panel of ΔLVEF < 11% after 26-weeks of empagliflozin treatment prediction

We performed tenfold cross-validation for the combined panel, and the panel (SIRT2, SIRT4, miR-182-5p, miR-302a-3p) demonstrated superior predictive performance for ΔLVEF < 11% (cross-validated AUC = 0.890) compared with individual biomarkers (Fig. 8). Among patients receiving empagliflozin, a cut-off value of 0.8316 provided 81% sensitivity and 90% specificity in predicting unfavourable outcome after 26-weeks of treatment. According to the multivariate logistic regression model, high baseline miR-182-5p and miR-302a-3p and high SIRT2 and SIRT4 expression panel was an independent predictor of ΔLVEF < 11% after 26-weeks of treatment (OR: 18.703; 95% CI, 5.78–60.49; $p < 0.0001$). Ezetimibe was also found to be a significant predictor of an unfavorable outcome after 26 weeks of empagliflozin treatment (OR: 0.138; 95% CI: 0.03–0.61; $p = 0.009$). Moreover, Spearman correlation analysis was employed to assess the relationship between ΔLVEF and the biomarker levels. A weak-to-moderate correlation was observed between ΔLVEF and SIRT2, SIRT4, miR-182-5p, miR-302a-3p (R = -0.536, R = -0.386, R = -0.433, R = -0.386, respectively, all $p < 0.0001$) (Table 4).

#### NT-proBNP association with ΔLVEF < 11% after 26-weeks of treatment post AMI

Based on NT-proBNP levels, there was no significant difference between the ΔLVEF < 11% and ΔLVEF ≥ 11% groups at baseline, either in the whole cohort or on drug comparison (Fig. 9A, B) (data not shown). Importantly, after 26-weeks of treatment, when we look at the drug effect on the NT-proBNP, there was no significant difference in the placebo group (Fig. 9B) (data not shown).

#### Machine learning based analysis

Efficient classification of clinical data, especially regarding adverse drug responses, is crucial for timely diagnosis and effective decision-making. Delays in assessment can lead to increased costs and health risks. ML enhances predictive performance, making analysis more efficient. In this study, we employed SHapley Additive exPlanations (SHAP) to improve interpretability and identify key features associated with drug response. SHAP enhances ML explainability by assigning Shapley values, a game-theory-based approach that quantifies each feature's impact on model predictions. Supervised ML techniques like SHAP effectively capture complex interactions and non-linear associations between variables.

Here, SHAP was used to evaluate the functions of laboratory and demographic data, providing valuable insights into predictive factors influencing patient outcomes. SHAP analysis calculated the contribution of each feature to the prediction indicating their importance. The SHAP

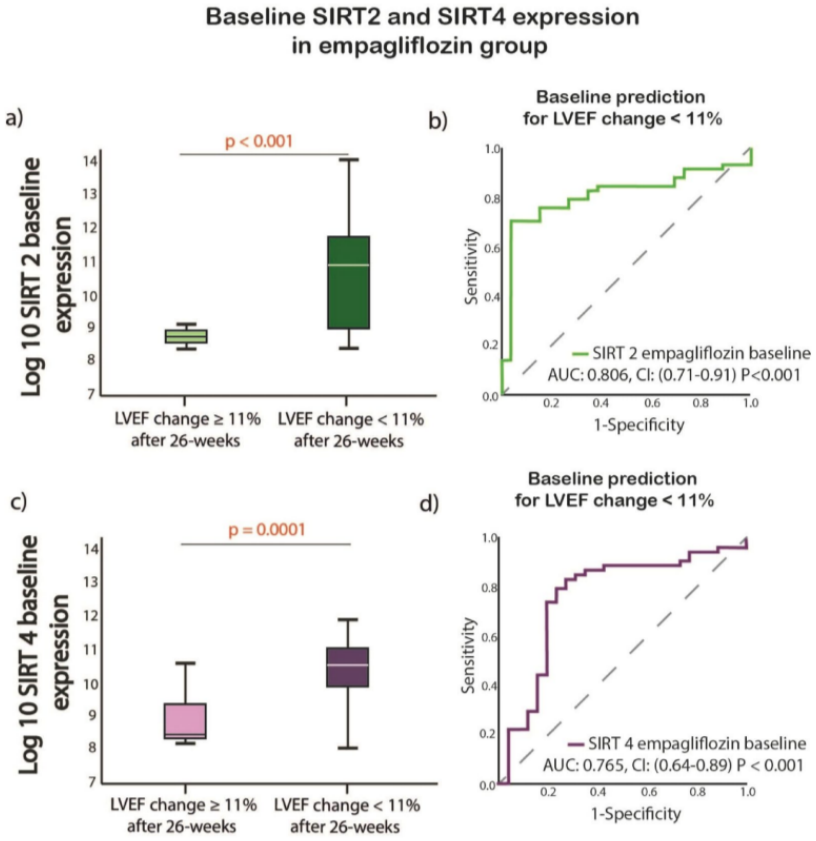



**Fig. 5** Baseline levels of box-plots and ROC curves: **a** SIRT2 baseline box-plots for outcome based on ΔLVEF; **b** SIRT2 baseline ROC curve for prediction of ΔLVEF < 11%; c) SIRT4 baseline box-plots for outcome based on ΔLVEF; d) SIRT4 baseline ROC curve for prediction of ΔLVEF < 11%

values we acquired enabled us to understand how having a particular value for a specific feature influences the prediction, as opposed to what the prediction would be with a baseline value for that feature. This method helps with the interpretability problem and can be used to improve the classification models (Fig. 10). Top features for laboratory data were panel filed (high score associated with ΔLVEF < 11%), SIRT2, miR-302, SIRT4 and SIRT5 before treatment. We also observed the influence of troponin. For demographic data, the key parameters were LVEF, age, and hypertension.

## Discussion

### Sirtuin pathways

In the multivariate logistic regression model, we found high baseline levels of SIRT2 and SIRT4 as independent predictors of ΔLVEF < 11%. Our findings align with previous research suggesting that elevated levels of SIRT2 and SIRT4 adversely impact cardiac function [47]. SIRT4 exacerbates angiotensin II (Ang II)-induced cardiac hypertrophy by inhibiting manganese superoxide dismutase (MnSOD) activity. MnSOD is an important antioxidant enzyme in the cardiac tissue, and inhibition of MnSOD via SIRT4 can cause an increase in oxidative stress, which thereby promotes the hypertrophic growth of cardiomyocytes and contributes to the progression of HF [47].

Additionally, SIRT4 may act as an inhibitor of glutamate dehydrogenase (GDH). GDH plays a crucial role in amino acid-stimulated insulin secretion by converting glutamate into α-ketoglutarate, thereby linking amino acid metabolism to the tricarboxylic acid cycle. By inhibiting GDH, SIRT4 reduces the availability of α-ketoglutarate, thus dampening insulin secretion in response to amino acids. This action of SIRT4 is shown

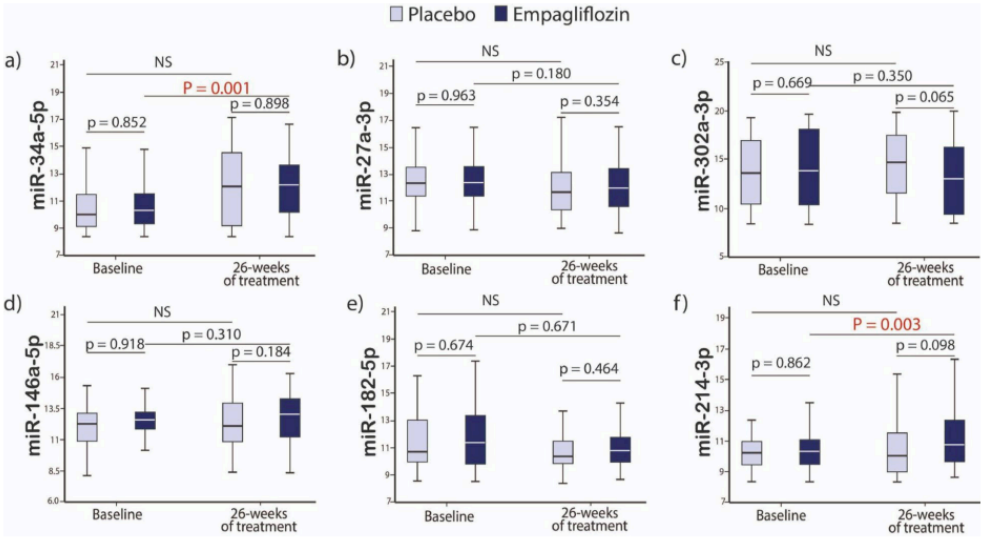



**Fig. 6** Boxplots for miRNAs expression between the treatments and time proportional changes regarding **a** miR-34a-5p; **b** miR-27a-3p; **c** miR-302a-3p; **d** miR-146a-5p; **e** miR-182-5p; **f** miR-214-3p. Un-paired data was calculated with Mann Whitney U test, Paired data was calculated by Wilcoxon test. All miRNA expression is presented by LOG10

to counteract the beneficial effects of caloric restriction [48].

Complementing this, the study by Zheng et al. found that the patients with increased plasma SIRT2 levels had higher percentage of HFrEF and higher level of brain natriuretic peptide (BNP). Additionally, they showed that they had a higher risk of MACE and HF after 1 year post-AMI [49]. We observed a significantly higher expression of SIRT6 in the empagliflozin group in comparison to placebo after 26-weeks of treatment highlighting the cardioprotective role of these sirtuins. Previously, Wang et al. described protective effects of SIRT6 after AMI by activating the AMPK-FoxO3a pathway, increasing antioxidant defenses and reducing oxidative stress in cardiomyocytes [50]. Furthermore, SIRT6 promotes CHMP2B degradation via the FoxO1-Atrogin-1 pathway, preventing autophagic dysfunction and mitigating aging-related myocardial damage [51].

In our study, we did not find significant alterations in SIRT1, SIRT3, SIRT5 and SIRT7 expression between the empagliflozin and placebo group. On the other hand, there are reports in the literature about their protective effect after AMI. The protective role of SIRT1 in various diseases is broadly described in the literature. Studies showed that SIRT1 can contribute to cardioprotection via several pathways, including energy/glucose metabolism, inflammation, and oxidative stress in cardiomyocytes. It mitigates ischemia–reperfusion injury by increasing antioxidant defenses, suppressing apoptosis, promoting autophagy, and minimizing oxidative stress [52, 53]. Porter et al. demonstrated that SIRT3 deficiency exacerbates ischemia–reperfusion injury, particularly in aged hearts, by impairing mitochondrial complex I (Cx I) activity and reducing MnSOD activity, both of which are key to maintaining cellular bioenergetics and antioxidant defenses. Additionally, SIRT3 regulates mitochondrial protein acetylation, and its downregulation in aged or SIRT3-deficient hearts leads to increased protein acetylation, which therefore can result in higher susceptibility to cardiac damage [54]. Additionally, SIRT5 was described as a protective factor in AMI by promoting autophagy and reducing apoptosis through its desuccinylation activity. Specifically, SIRT5 directly targets TOM1 at the K48 site. It was shown that SIRT5 and TOM1 genes are in regulation of autophagic processes during ischemia–reperfusion injury. In both in vitro and in vivo models, the overexpression of SIRT5 alleviated myocardial damage, demonstrating its potential as a therapeutic target for AMI [55].

Our study identifies elevated baseline SIRT2 and SIRT4 levels as independent predictors of poor LVEF recovery ($\Delta$LVEF < 11%), suggesting their adverse role in cardiac remodeling post-AMI. Along with previous findings highlight the complex, isoform-specific roles of sirtuins in AMI recovery and suggest that targeting the SIRT2 and SIRT4 pathways could refine drug response strategies for HF prevention.

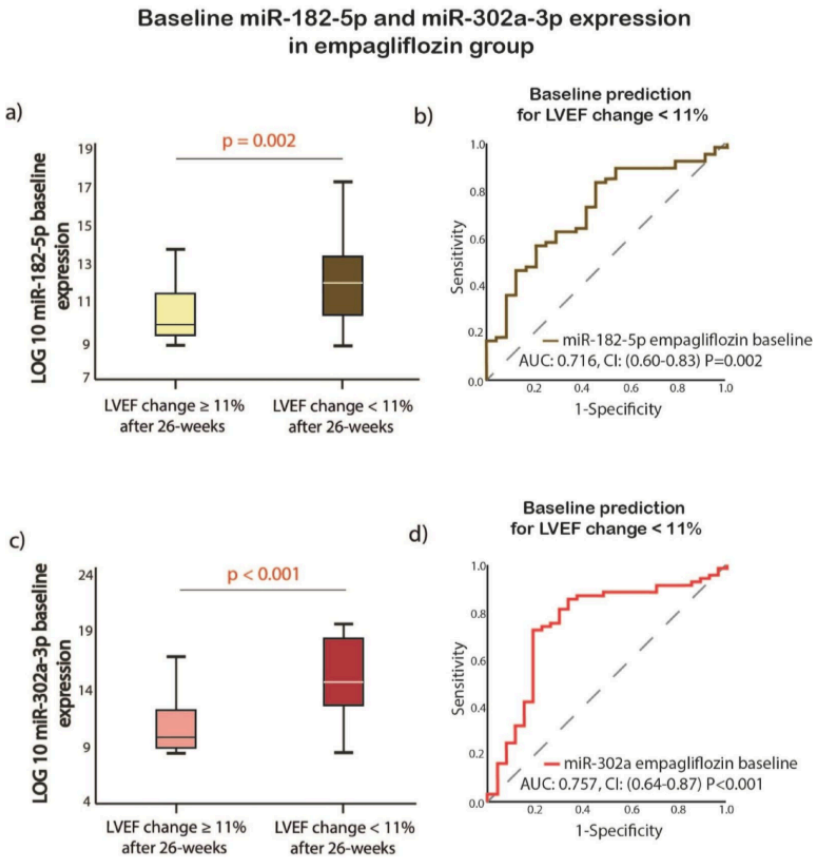



**Fig. 7** Baseline levels of box-plots and ROC curves: **a** miR-182-5p baseline box-plots for outcome based on ΔLVEF **b** miR-182-5p baseline ROC curve for prediction of ΔLVEF < 11% **c** miR-302a-3p baseline box-plots for outcome based on ΔLVEF **d** miR-302a-3p baseline ROC curve for prediction of ΔLVEF < 11%

**miRNAs pathways**

In our study we found that miR-182-5p and miR-302a-3p stand as predictive biomarkers of unfavourable ΔLVEF. In the literature we can find that the miR-182-5p exacerbates myocardial injury during AMI by promoting apoptosis and impairing myocardial cell viability. Overexpression of miR-182-5p leads to decreased expression of anti-apoptotic proteins like Bcl-2 and increased levels of pro-apoptotic markers such as Bax, Bnip3, and caspase-3/7, contributing to elevated cell death under hypoxic conditions. Conversely, inhibition of miR-182-5p enhances cell survival, suggesting its potential as a therapeutic strategy to mitigate myocardial damage and improve cardiac outcomes during ischemia [56]. Furthermore, the miR-302a-3p exacerbates myocardial ischemia–reperfusion injury by suppressing mitophagy through direct targeting of FOXO3. Its overexpression leads to mitochondrial dysfunction, increased oxidative stress, and enhanced apoptosis in cardiomyocytes. Inhibition of miR-302a-3p restores FOXO3 levels, promoting mitophagy, reducing oxidative stress, and improving mitochondrial function, making it a potential therapeutic target for mitigating myocardial injury during ischemia–reperfusion [57]. Additionally, by suppressing miR-302 activity, antagomiR-302 restores Mcl-1 expression, thereby limiting the activation of proapoptotic pathways and reducing cardiomyocyte apoptosis. The observed cardiomyocyte rescue under hypoxia/reoxygenation conditions suggests that antagomiR-302 may represent a promising strategy to protect the myocardium against ischemia-induced injury and to improve cardiac survival and function [58].

We did not detect statistically significant changes in the expression of miRNAs predicted in the bioinformatic

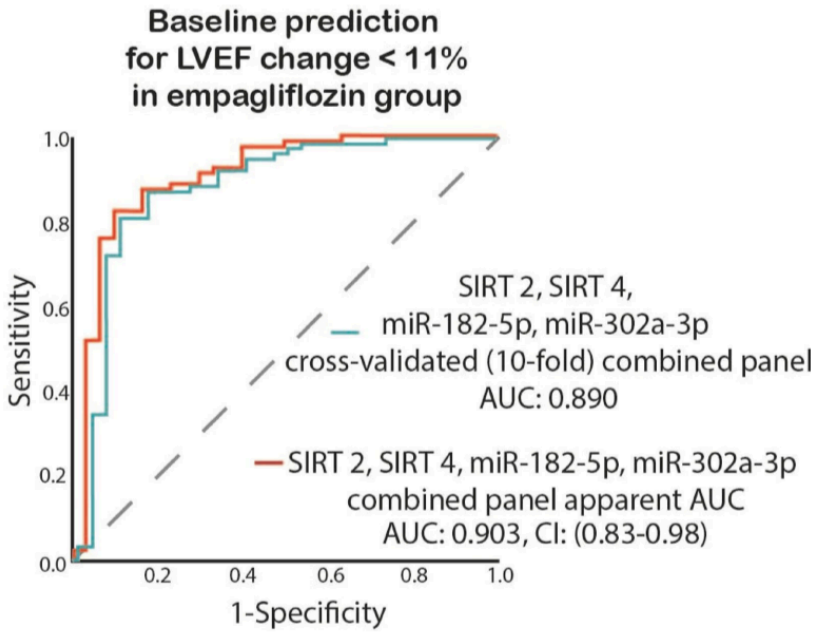



**Fig. 8** Combined panel ROC curves. Combined panel of parameters (high SIRT2, high SIRT4, high miR-182-5p, and high miR-302a-3p) baseline ROC curve for ΔLVEF < 11% after 26-weeks of treatment with empagliflozin. Abbreviations: AUC, area under the curve; CI, confidence interval

**Table 4** Multivariate logistic regression model for prediction of ΔLVEF < 11% after 26-weeks of empagliflozin treatment by high baseline combined high SIRT2, SIRT4, high miR-182-5p and high miR-302a-3p along with clinical variables at the baseline

| Variable | OR | 95% CI | | p-value |
|---|---|---|---|---|
| | | Lower | Upper | |
| Combined panel (SIRT2, SIRT4, miR-182-5p, miR-302a-3p) baseline | 18.703 | 5.783 | 60.492 | **< 0.0001** |
| Gender (female) | 0.530 | 0.113 | 2.494 | 0.557 |
| Age (years) | 1.062 | 0.994 | 1.135 | 0.074 |
| Hypertension (%) | 0.480 | 0.140 | 1.648 | 0.244 |
| BMI | 0.996 | 0.861 | 1.151 | 0.952 |
| Ezetimibe | 0.138 | 0.031 | 0.613 | **0.009** |

Abbreviations: CI, confidence interval; eGFR, estimated glomerular filtration rate; OR, odds ratio; SIRT, sirtuin

analysis namely miR-34a-5p, miR-27a-3p, miR-146a-5p, but those miRNAs are described in the ischemic injury pathways regarding MI. MiR-34a plays a significant role in AMI by negatively regulating SIRT1. It can lead to increased oxidative stress, apoptosis, and myocardial injury. Its overexpression exacerbates ischemia–reperfusion injury by further downregulating SIRT1, diminishing its protective effects on cellular survival and stress resistance. Conversely, inhibition of miR-34a restores SIRT1 expression, can increase cardiomyocyte viability, and improve cardiac function [59]. Similarly, Yang et al. showed that the miR-34a/SIRT1 axis modulates oxidative stress and cardiomyocyte survival, with resveratrol shown to mitigate injury by suppressing miR-34a and restoring SIRT1 levels [60]. Next, the miR-27 plays a role in AMI by negatively regulating metabolic processes in cardiomyocytes under hypoxic conditions, leading to impaired ATP consumption, decreased oxidative metabolism, and increased glycolysis. It targets and downregulates PPARγ, a key factor in lipid metabolism, which contributes to energy imbalances during ischemic conditions. Additionally, inhibition of miR-27 has been shown to partially restore the expression of SIRT1. Increased SIRT1 promoted cell survival and improved metabolic function in cardiomyocytes under hypoxic stress [61]. What is more, the miR-146a-5p plays a key role in AMI by regulating inflammation and apoptosis in cardiac cells. It is upregulated in ischemic heart disease and has been shown to be an independent predictor of major adverse cardiac events (MACE) in patients with STEMI. High levels of miR-146a-5p correlated with increased risk of MACE, making it a valuable biomarker for predicting long-term cardiovascular outcomes [62]. Another miRNA is miR-214,which contributes to cardiac hypertrophy by directly targeting SIRT3, a key mitochondrial regulator, leading

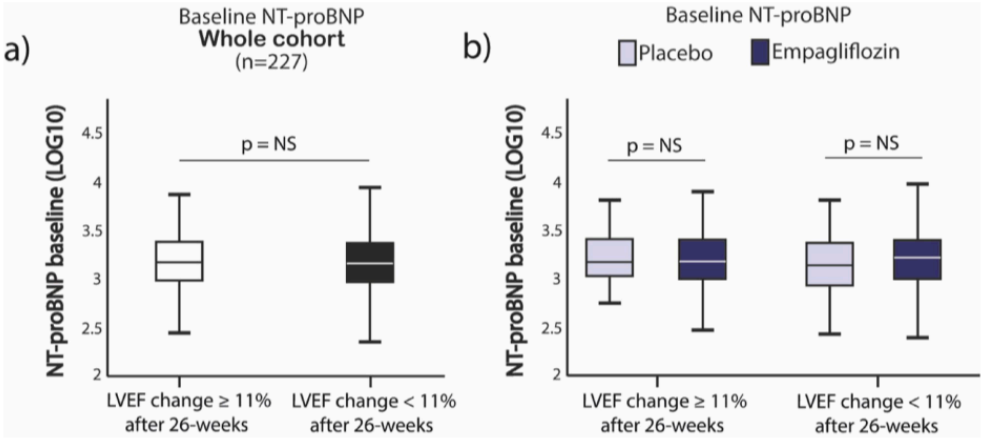



**Fig. 9** NT-proBNP changes based on the ΔLVEF between the empagliflozin and placebo groups and between the baseline and after 26-weeks. NT-proBNP concentration levels are presented by LOG10

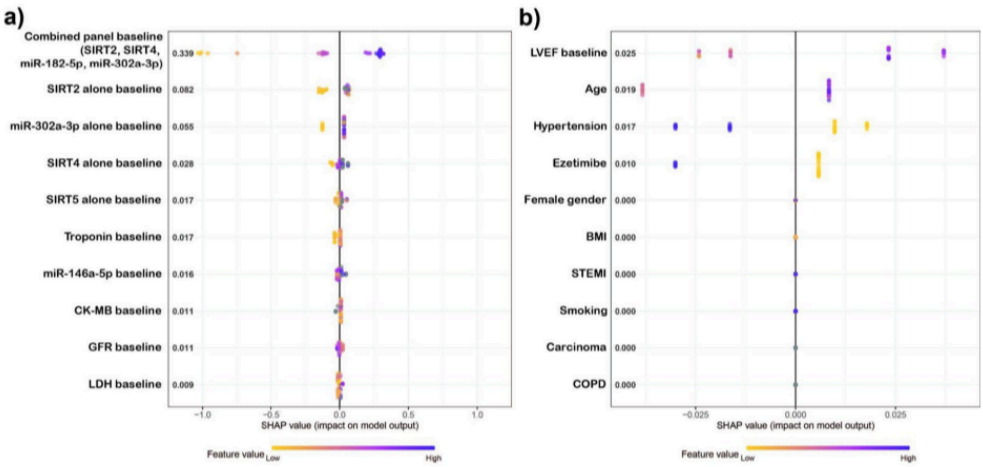



**Fig. 10** Results of SHAP method applied by using XGBoost classifier to the panel; **a)** laboratory and **b)** demographic part of clinical data. Results show the contribution locally per sample of each feature to the prediction and the most important features associated with the ΔLVEF < 11% outcome. The features with highest importance for a given outcome are localized on the top of the graph

to mitochondrial dysfunction and energy metabolism impairments. The study shows that the overexpression of miR-214 decreases SIRT3 levels, resulting in extensive mitochondrial damage and hypertrophy, while the inhibition of miR-214 restores SIRT3 expression and improves mitochondrial morphology and function. Therefore, the study suggested that miR-214 suppression may serve as a therapeutic strategy for mitigating cardiac hypertrophy by preserving SIRT3 activity [63].

Taken together, miR-182-5p and miR-302a-3p emerged as predictors of MI recovery, with mechanistic regulates to apoptosis (Bcl-2/Bax) and mitochondrial dysfunction (FOXO3). Though other predicted miRNAs (e.g., miR-34a, miR-146a-5p) showed no significant changes, their known roles in ischemic injury warrant further study for combinatorial biomarker or therapeutic applications.

### Clinical variables and treatment at the baseline

We observed that patients who had been receiving ezetimibe prior to MI were more likely to exhibit a favourable ΔLVEF. Given the statistically significant difference in ezetimibe use between patients with favourable and

unfavourable outcomes, we included ezetimibe treatment in the multivariate model. Consistently, Sohn et al. reported that a combined statin-ezetimibe regimen was associated with a lower risk of cardiovascular complications compared with statin monotherapy during a 5.5-year follow-up [64]. Furthermore, a systematic review by Zhan et al. demonstrated that the addition of ezetimibe to statins significantly reduced the risk of non-fatal myocardial infarction [65]. What is more, patients with pre-existing hypertension demonstrated better post-MI outcomes compared to normotensive individuals, likely attributable to optimized blood pressure control mitigating cardiovascular complication risks [66]. These findings show the potential benefits of lipid and blood pressure management as beneficial in high-risk populations.

#### In silico annotation strategy and limitations of the study

Our miRNA and gene-tissue annotations relied on curated in silico resources (TISSUES 2.0 and multiMiR). TISSUES integrates transcriptomic, proteomic and literature evidence into a unified confidence score (0–1). We used ≥ 0.5 to retain only medium-to-high reliability associations of miRNA while avoiding over-restriction that would discard known blood/plasma markers. Because plasma miRNAs can originate from multiple blood cell types and peripheral tissues, the database score does not imply strict cell-type specificity or disease-context regulation. Likewise, multiMiR collates predictions from multiple algorithms; individual predictions may vary across methods and not all pairs have experimental validation. To minimize bias, we: (i) restricted tissue annotation to blood/plasma entries, (ii) required consensus ranking (top 20%) across 14 databases for miRNA-target relationships, and (iii) interpreted miRNA-gene interaction findings as hypothesis-generating rather than confirmatory. These choices prioritize specificity without unduly narrowing the analyzable space. In this paper we therefore present the computational annotations as contextual support for the experimental results. While we performed gene expression analysis to assess sirtuins' mRNA levels, corresponding protein quantification was not conducted due to the limited volume of available plasma samples and the lack of validated, high-sensitivity assays for circulating SIRT proteins. Additionally, all participants in this study were of Caucasian ethnicity, as recruitment was conducted exclusively in Austria, Europe. This may limit the generalizability of the findings to more diverse populations, underscoring the need for future studies to include participants of different ethnic backgrounds. What is more, a limited number of female participants, restricting sex-specific analyses. Furthermore, the lack of external validation in an independent cohort limits the generalizability of our findings. Although the biomarker panel demonstrated a strong association with the outcome, as indicated by a large odds ratio and wide confidence interval, these results should be interpreted with caution. Independent validation is essential before this panel can be considered for clinical application. Another limitation of our study is the absence of functional assays and mechanistic in vitro or in vivo experiments to confirm the causal role of the identified miRNAs. These analyses remain essential future steps to validate our findings. An additional limitation is that while bioinformatic analyses predicted associations between MI/HF and modulation of SIRT1, SIRT3, SIRT5, and SIRT7 along with miR-34a-5p, miR-27a-3p, miR-146a-5p, and miR-214-3p, our laboratory analyses failed to detect significant differences in these markers. Notably, despite SIRT1 being the most extensively studied sirtuin in CV contexts, we observed no significant alterations in its expression levels. Sirtuins are inherently difficult to detect in plasma, and while SIRT1-5 reached > 70% detection, the lower rates for SIRT6 and SIRT7 (65–67%) reduce their robustness as biomarkers and therefore they were not included in the multivariate analysis. A final and the most important limitation is that our clinical trial evaluated only empagliflozin among SGLT2 inhibitors. Consequently, we cannot determine whether patients with suboptimal response to empagliflozin might benefit from alternative SGLT2 inhibitors when initiated early post-AMI. These findings underscore the need for future studies to comparatively evaluate multiple SGLT2 inhibitors in the AMI setting.

## Conclusions

Our findings highlight the significant role of empagliflozin in modulating sirtuin and its regulatory miRNAs expression may contribute to its cardioprotective effects in patients with AMI. Importantly, for the first time, our findings showed that baseline levels of SIRT2, SIRT4, miR-182-5p, and miR-302a-3p can emerge as independent predictors of ΔLVEF < 11% after 26-weeks of treatment, which suggests the potential of those markers as stratification of responders and non-responders to empagliflozin in patients with AMI. Moreover, the combined panel of high SIRT2, high SIRT4, high miR-182-5p, and high miR-302a-3p demonstrated the highest predictive accuracy compared to individual markers. These findings provide valuable insights into the epigenetic modifications associated with SGLT2 inhibitors and suggest that genomic characterization may help stratify responders and non-responders to empagliflozin.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12933-025-03013-y.

Supplementary Material 1

**Acknowledgements**
This work was written by the members (ANS, CE, ZW, JP, JS-M, DvL, HS, MP) of the International and Intercontinental Cardiovascular and Cardiometabolic Research Team (I-COMET; www.icomet.science).

**Institutional Review Board Statement**
The study was conducted in accordance with the Declaration of Helsinki and was approved by the relevant regulatory authorities, by the Ethics Committee of the Medical University of Graz, Austria (EK 29–179 ex 16/17; EudraCT 2016-004591-22) and registered on ClinicalTrials.gov (NCT03087773).

**Author contributions**
Writing-original draft preparation, ANS, CE, ZW, MP; writing-review and editing, ANS, CE, ZW, JP, JS-M, DvL, HS, MP; bioinformatic and machine learning analysis, ZW; visualization, CE, ZW; supervision, CE, MP; laboratory analysis, ANS, SA; Data preparation, ANS, CE, HS. All authors have read and agreed to the published version of the manuscript.

**Funding**
ANS was supported financially as part of the research grant from Polish National Science Center (NCN), Poland (grant number 2022/45/N/NZ7/0246) and MP by the Polish Medical Research Agency (ABM) (grant number 2019/ABM/01/00037). HS and his research is supported by the Austrian Science Fund (FWF) (PIN8074224) and the Horizon Europe project PoCCardio (grant number 101095432). DvL is supported by the Austrian Science Fund (FWF) Grant KLIF-1155-B.

**Data availability**
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing interests**
Authors declare no conflict of interest related to this work.

**Informed consent**
Informed consent was obtained from all subjects involved in the study and written informed consent has been obtained from the patient(s) to publish this paper.

**Author details**
[1]Department of Clinical and Experimental Pharmacology, Center for Preclinical Research and Technology (CePT), Medical University of Warsaw, Banacha 1B, 02-097 Warsaw, Poland
[2]Department of Diabetology and Internal Medicine, Medical University of Warsaw, Warsaw, Poland
[3]Doctoral School, Medical University of Warsaw, Warsaw, Poland
[4]Genomics Core Facility, Centre of New Technologies, University of Warsaw, Warsaw, Poland
[5]Department of Neurochemistry, Institute of Psychiatry and Neurology, Warsaw, Poland
[6]Department of Internal Medicine II, Division of Cardiology, Medical University of Vienna, 1090 Vienna, Austria
[7]Department of Internal Medicine, Division of Cardiology, Medical University of Graz, Graz, Austria
[8]Division of Endocrinology and Diabetology, Cardiometabolic Trials Unit, Medical University of Graz, Graz, Austria

**Received: 11 June 2025 / Accepted: 5 November 2025**
Published online: 29 December 2025

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## Publisher's Note

## 6.2. Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health

npj | systems biology and applications

Article

Published in partnership with the Systems Biology Institute

https://doi.org/10.1038/s41540-025-00619-6

# Integrative gene-metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health



Zofia Wicik[1,2], Anna Nowak-Szwed[1,3,4], Ceren Eyileten[1,5], Harald Sourij[6], Dirk von Lewinski[7], Svjatoslavs Kistkins[8,9], Joanna Borkowska[10] & Marek Postuła[1] ✉

Glucagon-like peptide-1 (GLP-1) is a hormone known for its critical functions in managing blood sugar and offering cardiovascular benefits. Our study focuses on Glucagon Like Peptide 1 Receptor (GLP1R) agonists that act beyond glycemic control in cardiovascular and metabolic health. A comprehensive bioinformatic analysis was conducted, incorporating GLP1R, Gastric Inhibitory Polypeptide Receptor (GIPR), Gastric Inhibitory Polypeptide (GIP) and glucagon receptor (GCGR) to assess the effects of GLP1R agonists on gene and metabolite interactions. Interaction network analysis revealed 130 common genes among GLP1R, GLP1R/GIPR, GLP1R/GIP, and GLP1R/GIPR/GCGR associated with diabetes-related processes, including obesity and hyperglycemia. Enriched terms related to cardiovascular diseases, such as hypertension, calcium regulation in cardiac cells, and amino acid accumulation-induced mTOR activation. We also observed enrichment in gene sets linked to longevity and less recognized terms like fatty liver disease. In GLP1R/GIP, behavior-related terms and gastric acid secretion were identified; GLP1R/GIPR/GCGR linked to fibrosarcoma, thought/speech disturbances, and adipogenesis. The metabolite-gene layer revealed enrichment in galactose metabolism, platelet homeostasis, and nitric-oxide pathways. We found that GLP1R agonists network-level associations are stronger with heart diseases than sodium-glucose co-transporter 2 inhibitors, suggesting greater therapeutic benefits. Integrating networks with metabolites highlighted key interactors and clarified GLP1R agonists' mechanisms and therapeutic potential.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone, secreted by intestinal L-cells, with levels rising after nutrient intake. When nutrient levels increase, the secretion of GLP-1 intensifies, leading to a significant elevation in its circulating levels[1]. The conventional functions of GLP-1 are associated with its capacity to boost insulin secretion in response to glucose stimulation. However, the recognized effects of GLP-1 have swiftly broadened by its ability to inhibit gastric emptying, glucagon secretion, stimulate weight loss and exhibit beneficial influence on the cardiovascular (CV) system. These unique abilities lead to the creation of various GLP-1 receptor agonists (GLP1RA) for the management of type 2 diabetes (T2D) and further the cardiometabolic syndrome[2].

Both glucagon and GLP-1 are derived from the GCG gene, which encodes the proglucagon precursor. It undergoes tissue-specific processing to yield several biologically active peptides. In pancreatic α-cells,

[1]Department of Experimental and Clinical Pharmacology, Center for Preclinical Research and Technology CEPT, Medical University of Warsaw, Warsaw, Poland. [2]Department of Experimental and Clinical Neuroscience, Institute of Psychiatry and Neurology, Warsaw, Poland Sobieskiego 9. [3]Doctoral School, Medical University of Warsaw, Warsaw, Poland. [4]Department of Diabetology and Internal Medicine, University Clinical Centre, Medical University of Warsaw, Warsaw, Poland. [5]Genomics Core Facility, Centre of New Technologies, University of Warsaw, Warsaw, Poland. [6]Trials Unit for Interdisciplinary Metabolic Medicine, Division of Endocrinology and Diabetology, Medical University of Graz, Graz, Austria. [7]Division of Cardiology, Medical University of Graz, Graz, Austria. [8]Institute of Applied Computer Systems, Riga Technical University, Riga, Latvia. [9]Research Institute, Pauls Stradins Clinical University Hospital, Riga, Latvia. [10]Laboratory of Functional and Structural Genomics, Centre of New Technologies, Warsaw, Poland. ✉e-mail: mpostula@wum.edu.pl

proglucagon is cleaved to glucagon, a key hormone maintaining glucose homeostasis through stimulation of hepatic glucose output, preventing hypoglycemia in fasting. However, in the context of T2D, inappropriate secretion of glucagon contributes to hyperglycemia, exacerbating the metabolic dysfunction[3]. In contrast, intestinal L-cells generate GLP-1, which counteracts this by inhibiting glucagon secretion, as well as glucagon-like peptide-2 (GLP-2), which promotes intestinal growth and nutrient absorption[3]. Additional proglucagon-derived peptides, including glicentin and oxyntomodulin, contribute to gastrointestinal regulation and appetite control. These proglucagon-derived peptides exert their effects through distinct receptors: glucagon signals via the glucagon receptor (GCGR), GLP-1 via the GLP1R, and GLP-2 via the GLP-2 receptor (GLP2R). Oxyntomodulin acts as a dual agonist at both GCGR and GLP1R, while the actions of glicentin remain less well defined but are suggested to influence gastrointestinal physiology[4].

Gastric inhibitory polypeptide (GIP), another incretin hormone secreted by K-cells in the proximal small intestine, enhances insulin secretion in a glucose-dependent manner, similar to GLP-1. In addition, it regulates lipid metabolism by promoting fatty acid uptake and storage in adipose tissue. However, GIP's physiological response is often blunted in obesity and T2D, which may contribute to metabolic dysfunction[5]. Unlike GLP-1, GIP does not markedly influence satiety or gastric emptying, highlighting their functional divergence[6]. Importantly, while GLP-1 inhibits glucagon release to reduce hepatic glucose output, GIP can stimulate glucagon secretion under certain conditions, such as low glucose states, which may represent an adaptive mechanism to maintain glucose supply during fasting or stress[3]. Together with glucagon, these nuanced actions of GLP-1 and GIP illustrate the complexity of incretin crosstalk in maintaining metabolic homeostasis.

Class B1 G protein-coupled receptors (GPCRs) play a crucial role in regulating physiological functions, particularly glucose homeostasis and mechanism of action in cardiometabolic syndrome. This regulation is primarily mediated by the peptide hormones GLP-1, glucagon, and GIP, which activate these receptors, leading to a signaling cascade and the formation of an active receptor-G protein complex[7]. Additionally, intracellular signal transducers can activate GPCRs without external stimuli. This indicates that G proteins have an intrinsic role in this process. These receptors are significant drug targets for various conditions, including T2D, obesity, osteoporosis, migraines, CV diseases, and short bowel syndrome[8]. Pharmacological strategies targeting multiple pathways have shown synergistic benefits in metabolic disease models. One such strategy, triagonism, involves activating GLP-1, GIP, and GCGR, combining GLP-1's and GIP's effects on appetite suppression and insulin secretion with glucagon role in energy expenditure[9]. However, the specific role of the glucagon receptor activation has not been fully understood. Literature data shows that optimized tri-agonists normalize body weight in diet-induced obesity (DIO) in mice and increase energy expenditure more effectively than GLP1R mono-agonists or GLP1R/GIPR (Gastric inhibitory polypeptide receptor) co-agonists[9]. In our study we are exploring this tri-agonistic effect as well as the separate influence of pairs with GPL1R and GIP, GIPR and GCGR through analyzing GLP1R interaction networks and associated metabolomic data.

GLP-1 is secreted from intestinal L-cells in a nutrient-dependent manner and, through binding to the GLP1R[10]. Beyond its metabolic effects, GLP1R is expressed in the central nervous system, where its activation influences satiety and may exert neuroprotective effects[11,12]. GLP1RAs have shown broad cardiometabolic benefits. CV outcome trials demonstrated reductions in atherothrombotic events, especially in patients with established atherosclerosis[13]. Mechanistically, GLP1RAs improve cardiac function by enhancing contractility and reducing myocardial injury[14–16], exert vasodilatory and endothelial-protective effects, and display anti-inflammatory and anti-fibrotic actions that may limit adverse remodeling in heart failure (HF)[17]. They also improve glucose and lipid metabolism[18] and may protect renal function, contributing further to CV benefit[19]. Although most evidence concerns T2D and obesity, GLP1RAs are also being investigated in neurological disorders such as Parkinson's disease, where they may protect dopaminergic neurons and improve mitochondrial function, though current evidence remains preliminary[20]. The most frequent adverse effects include gastrointestinal symptoms, appetite loss, tachycardia, pancreatitis, and a possible risk of thyroid tumors, warranting careful monitoring[21–24].

Both GLP1R and GIPR regulate nutrient-stimulated insulin secretion, and GIP, like GLP-1, promotes insulin release while supporting β-cell proliferation and survival[25]. Recent studies indicate that central GIPR activation reduces body weight by suppressing food intake, whereas peripheral activation promotes weight loss via non-intake mechanisms[26]. Together, GLP-1, GIP, and their receptors play essential roles in postprandial glucose homeostasis[9]. Dual GLP1R/GIPR agonism, particularly when combined with GCGR activity, may enhance glycemic control and weight reduction. Preclinical data suggest that the most pronounced weight loss occurs when GCGR potency exceeds that of GLP1R or GIPR, supporting the concept of triple agonism as a promising therapeutic approach for obesity[9,27]. Beyond glucose regulation, GIP agonists have been shown to modulate lipid metabolism, improve dyslipidemia, and reduce atherogenesis in low-density lipoprotein (LDL) receptor knockout mice[28]. Combined treatment with GLP1R and GIPR agonists may result in improved metabolic and antidiabetic responses, with GIP indicating the potential of the agonist in CV health through its actions on lipid metabolism, insulin secretion, and cardioprotective properties[29].

Through binding to the GCGR, glucagon primarily acts on the liver to raise blood glucose via glycogenolysis and gluconeogenesis[30]. While endogenous glucagon prevents hypoglycemia, pharmacological GCGR agonism has attracted attention as a therapeutic strategy in obesity and T2D, as it can increase energy expenditure and reduce food intake despite its hyperglycemic properties[31].

Beyond metabolic regulation, glucagon has well-documented CV effects. GCGR activation in the heart increases heart rate and contractility through G protein-mediated stimulation of adenylyl cyclase and cAMP production in cardiomyocytes[32]. Animal studies show that glucagon enhances ventricular contractility while exerting antiarrhythmic effects on ventricular arrhythmias, likely via supraventricular mechanisms[33]. These findings suggest that glucagon may have dual roles, supporting cardiac output in acute settings while also modulating rhythm stability.

Taken together, GCGR agonism offers a promising yet complex strategy in cardiometabolic disease, as it promotes weight loss and energy expenditure while also increasing blood glucose, underscoring the need for dual- and triple-agonist approaches (GLP1R/GIPR/GCGR) that balance metabolic and CV effects. Given the widespread use of GLP1RAs in T2D, obesity, and CV comorbidities, their pleiotropic mechanisms remain to be clarified.

In this study, we used bioinformatic network analyses to (i) investigate cardioprotective pathways of GLP1RAs through interaction networks and metabolic data, and (ii) assess their combinations with SGLT2i and metformin to explore CV effects beyond glycemic control, (iii) investigate interactors that could play a role in longevity-relevant and major known signaling pathways.

## Results

### Workflow of performed analyses

In the present study we investigated two levels of interactions for agonists of the GLP1R, GIPR, and GCGR in following combinations: GLP1R (semaglutide), GLP1R/GIPR (tirzepatide), GLP1R/GIP and triple agonist GLP1R/GIPR/GCGR (retatrutide). We decided to include both GIPR and GIP in the analysis to better understand the effect of activation of related pathways. First level of interactions included direct interactors of analyzed nodes, while secondary interactions included first level interactions and all of their neighbors (Table 1). The workflow of bioinformatic analysis is shown in Fig. 1. Additionally, only for initial analysis (Venn diagrams), we included SGLT2i (empagliflozin, dapagliflozin) and metformin. Results of gene set enrichment analyses are available in Supplementary data 1.

Tissue-specific expression analysis aimed to identify the most affected tissues by highly expressed components of different versions of GLP1R networks: GLP1R, GLP1R/GIPR, GLP1R/GIP, GLP1R/GIPR/GCGR. We focused on the top 30% of genes with highest expression confidence within analyzed tissues. Analysis identified multiple tissues related to the gastrointestinal system and nervous system. Highest overexpression was associated with the paraventricular nucleus, responsible for motor output systems of the hypothalamus and gastric motility, especially by GLP1R/GIP. The second affected tissue was the area postrema. We also observed multiple nervous tissues related to the activity of gastrointestinal hormones regulating appetite (Fig. 2). An effect on the CV system was observed for the 2nd level interaction networks.

**Table 1 | Lists of analyzed targets/ drugs and associated genes for different levels of regulation**

| Target/ drug proxy | Regulation level | No. of genes (*n*) | Targets |
|---|---|---|---|
| GLP1R | Target | 1 | GLP1R |
| GLP1R | net1 | 130 | |
| GLP1R | net2 | 7348 | |
| GCG | Target | 1 | GCG |
| GCG | net1 | 326 | |
| GCG | net2 | 10587 | |
| GIPR | Target | 1 | GIPR |
| GIPR | net1 | 99 | |
| GIPR | net2 | 5426 | |
| GLP1R/GIPR | Target | 2 | GLP1R GIPR |
| GLP1R/GIPR | net1 | 179 | |
| GLP1R/GIPR | net2 | 8483 | |
| GLP1R/GIP | Target | 2 | GLP1R GIP |
| GLP1R/GIP | net1 | 193 | |
| GLP1R/GIP | net2 | 8263 | |
| GLP1R/ GIPR/GCGR | Target | 3 | GCGR GIPR GLP1R |
| GLP1R/ GIPR/GCGR | net1 | 202 | |
| GLP1R/ GIPR/GCGR | net2 | 9302 | |
| SGLT2 | Target | 1 | SLC5A2 |
| SGLT2 | net1 | 80 | |
| SGLT2 | net2 | 5192 | |
| Empagliflozin | Target | 2 | SLC5A1 SLC5A2 |
| Empagliflozin | net1 | 164 | |
| Empagliflozin | net2 | 7912 | |
| Dapagliflozin | Target | 4 | SLC5A1 SLC5A2 SLC5A11 SLC5A4 |
| Dapagliflozin | net1 | 180 | |
| Dapagliflozin | net2 | 8433 | |
| Ertugliflozin | Target | 3 | SLC5A1 SLC5A2 SLC5A4 |
| Ertugliflozin | net1 | 171 | |
| Ertugliflozin | net2 | 8062 | |
| Metformin | Target | 8 | SLC22A2 SLC22A1 ABCC3 ABCC4 ABCB11 DHFR DPP4 SLC47A1 |
| Metformin | net1 | 663 | |
| Metformin | net2 | 14291 | |

### Interaction network analysis

To pinpoint the top GLP1R interactors, we performed interaction network analysis. We integrated genes from primary and secondary networks with related compounds, with interactions shared between them. We identified GLP1R, GCG, AKT1, INS, POMC, LEP, PPARG, GHRL, PPARA, ADCY6, NPY, MAPK3, VIP and NOS3 as the top interactors with highest connectivity associated with MI and HF. Metabolites most frequently mapped to GLP1R first-level neighbors included ATP and and alpha-D-Glucose, top lipid was 8-HETE. Metabolite interaction network was strongly affected by GCK which is the 3rd interactor in GLP1R interactome (Fig. 3A).

Interaction networks focused on gene-metabolite interactions showed the highest potential of GCK in the regulation of important metabolites. Top metabolites affected by 1st level interactors and 1st and second levels of interactors of the GLP1R network were D-Glucose and alpha-D-Glucose (Fig. 3B).

### Gene set overlap and enrichment analysis

Gene set overlap analysis allowed us to identify the number of genes from 1st level interactors and both levels of interactors that are common between analyzed drugs (Fig. 4). We found 32 genes shared between GLP1RA, SGLT2i and metformin (ACE, ADIPOQ, ALB, CCK, DPP4, ENSP00000387760, FAP, FFAR1, FFAR4, GCG, GCK, GHRL, GIP, GLP1R, GLP2R, GNAT3, GPR119, IAPP, INS, LEP, MGAM, NEUROG3, PPARG, PYY, REN, SI, SLC2A2, SLC5A1, SLC5A2, SLC5A4, TAS1R2, TAS1R3)

In order to identify phenotypes, processes, and pathways associated with the analyzed drugs, we performed gene enrichment analysis on the selected gene lists. First, we analyzed the primary interaction networks, and later, the secondary ones (Fig. 4A, B). Due to data presentation limitations, only the top 5 affected processes for each analysis are presented.

**Overlap between interaction networks**. The analysis of the overlap between different GLP1RAs revealed 130 common genes (Fig. 4C). These top 130 genes were significantly associated with diabetes-related processes, including obesity and hyperglycemia. Additionally, ontological terms related to CVD were observed, including hypertension (ACE, ADIPOQ, ADRB2, ALB, CASP3, CAV1, CRH, DPP4, FOS, GHRL, GNB3, INS, IRS1, LEP, LEPR, MGAM, NOS3, NPPA, NPY, POMC, PPARA, PPARG, PTH, REN, SST, VIP), calcium regulation in the cardiac cell (ADCY5-6-8, ADRB2, ARRB1-2, GNAQ, GNAS, GNB1-5, GNG11-13, GNG2-8, GNGT1), and process "failing heart cytosolic accumulation of amino acids that can promote the activation of mTOR" (AKT1, IRS1, IRS2). Furthermore, sets of genes from the Disgenet database related to MI and HF were observed. Additionally, 26 genes associated with hypertension were also significantly involved in the adipocytokine pathway ($p$ adj = 7.741e−10), maltose and fructose metabolism ($p$ adj = 0.01746), C-reactive protein (CRP) ($p$ = 0.003519), and inflammation ($p$ = 0.007776).

The analysis revealed enrichment of gene sets associated with longevity. In addition to well-known terms, there were also associations with terms related to fatty liver disease (ADIPOQ, AKT1, ALB, DPP4, FGF21, FOXO1, GCG, INS, IRS1, IRS2, LEP, LEPR, PPARA, PPARG, PPARGC1A), chemokine signaling, and sirtuin interactome. A secondary network of overlapping genes showed enrichment in various cancers, including Breast Carcinoma and Central carbon metabolism in cancer. Additionally, enrichment related to obesity and pathways associated with multiple CVDs phenotypes, such as coronary artery disease and hypertension, were observed. Terms related to inflammatory processes, such as arthritis and inflammation, were also found (Fig. 4D).

**GLP1R/GIP interaction network**. The primary GLP1R/GIP network processes include terms related to behavior such as hyperactive behavior and panic disorder (NPS, ADORA2A, CCKBR, GRP, PTK7). Additionally, there is observed enrichment of hypercalcemia (STC1, GAST, PTHLH), urticaria (severe eczema) (NPS, CYSLTR2,

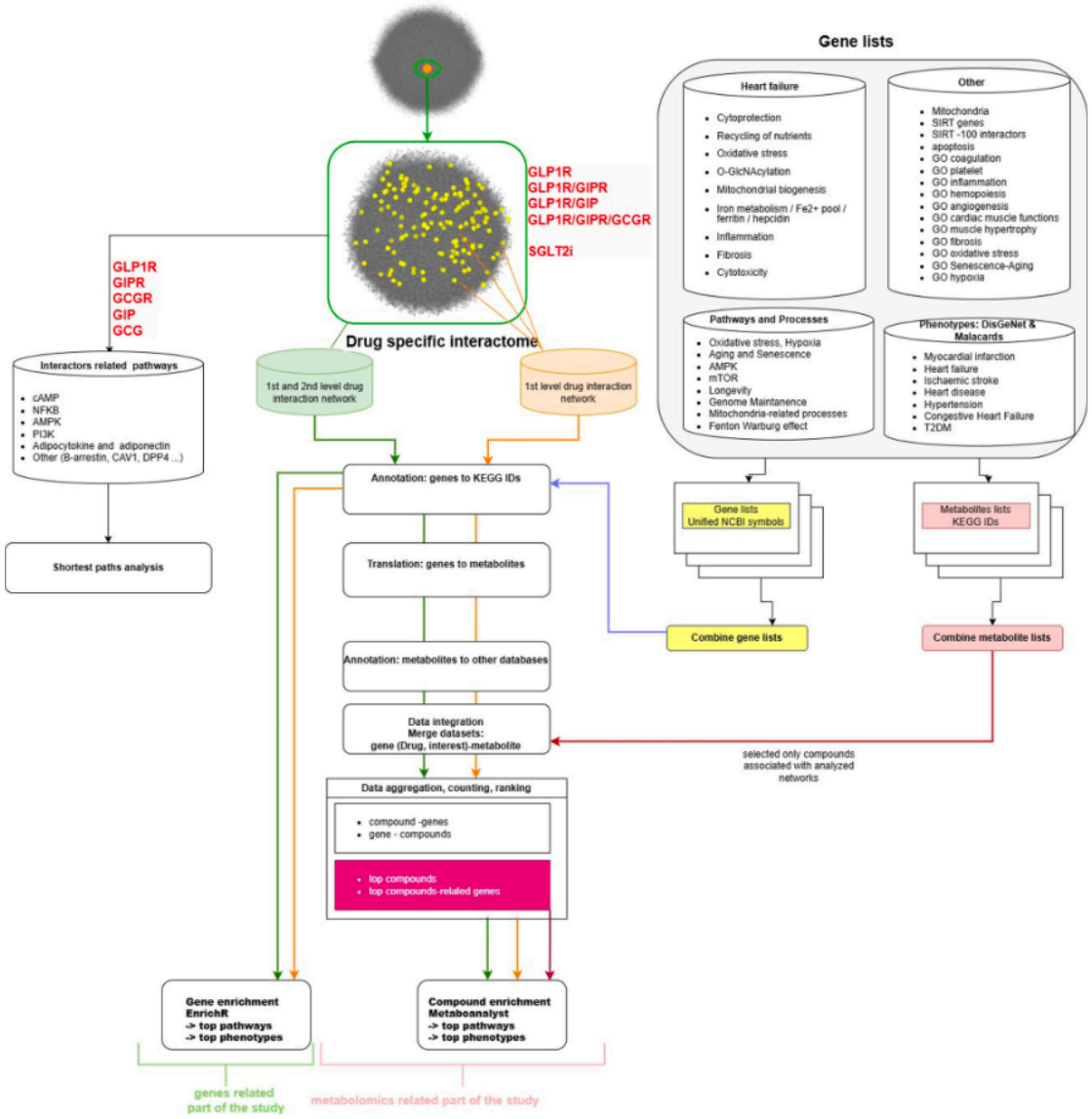



**Fig. 1 | Workflow of bioinformatic analysis.** Figure was created using open source https://app.diagrams.net/ webpage.

TAC1), and secretion of hydrochloric acid in parietal cells (CCKBR, GAST). Processes specific to the secondary network of tirzepatide include CVD-related congenital heart disease and Ebstein anomaly (Fig. 4D).

**GLP1R/GIPR/GCGR interaction network.** The processes specific to the GLP1R/GIPR/GCGR network included fibrosarcoma (STC1), thinking and speaking disturbances, and adipogenesis-related signaling pathways. On the other hand, processes specific to the secondary network involved 30 genes related to the Parkin-Ubiquitin Proteasomal System pathway (PSMD10, PSMD12, PSMD11, PSMD14, TUBAL3, HSPA1L, PSMD13, HSPA6, UBE2J2, HSPA14, PSMD8, PSMD7, PSMD4, PSMC3, PSMC4, PSMC1, PSMD3, PSMC2, PSMD1, UBA1), Striated Muscle Contraction Pathway, genes associated with Genome Maintenance, oxidative stress, and hypoxia-related terms (Fig. 4C).

**GLP1R/GIPR interaction network.** The GLP1R/GIPR network did not regulate unique genes, but it shared multiple processes with the GLP1R/GIP and GLP1R/GIPR/GCGR related networks. Ontological terms shared with the primary network of GLP1R/GIPR/GCGR included body mass index, metabolic syndrome, and T2D. For the secondary network, there were multiple terms related to mitochondrial activity, including mitochondrial diseases and failing heart impaired mitochondrial function.

All three drug networks shared Acth-Independent macronodular adrenal hyperplasia (GIPR, HTR4), mood disorders (PSMB6, PLXNA3), and lipid storage disease. For the secondary network, there was 3-Methylcrotonyl-CoA carboxylase deficiency (MCCC2, CGB8, CGB7, MCCC1), amino acid metabolism, and pyrimidine metabolism.

Both the GLP1R/GIP network and the GLP1R/GIPR/GCGR network shared primary ciliary dyskinesia, but different genes. More specifically, for

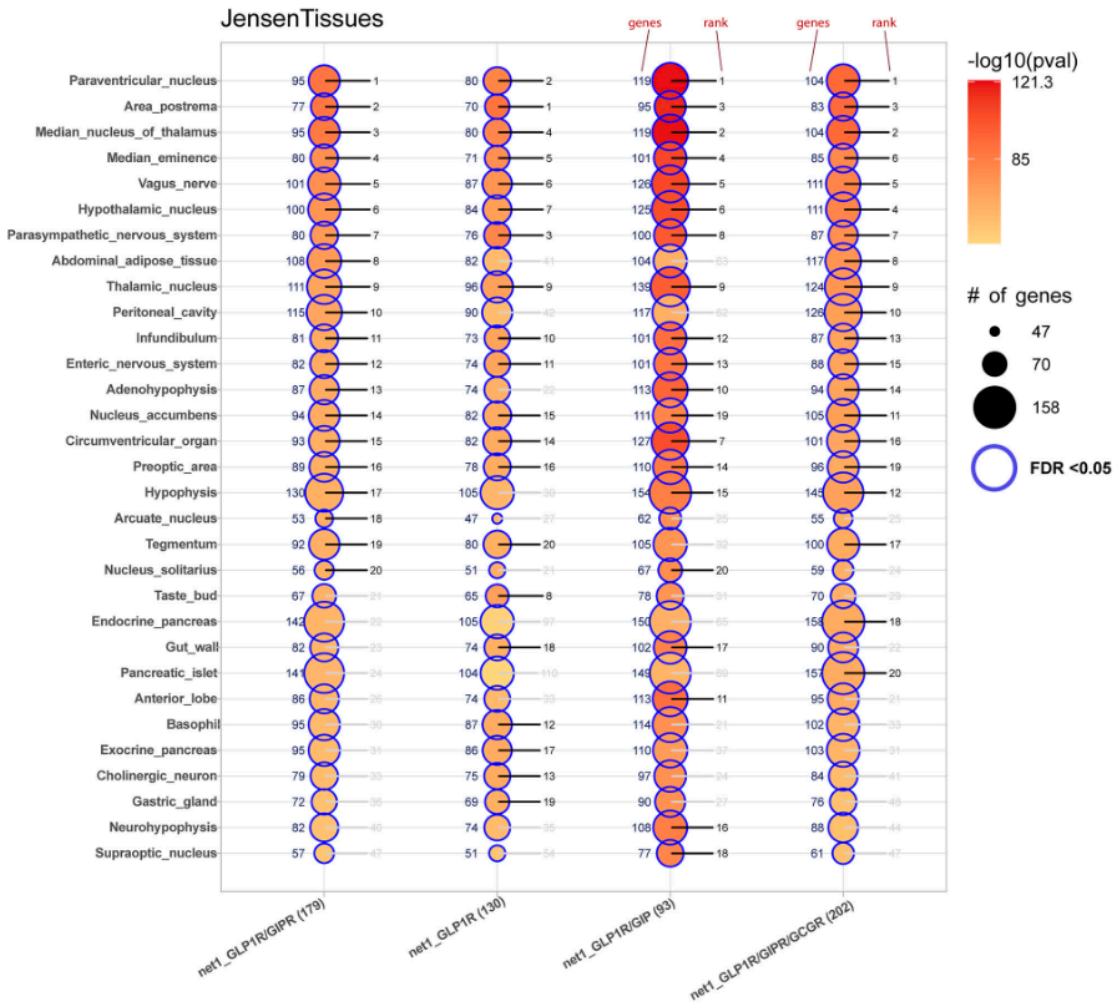



**Fig. 2 | Enrichment analysis of "tissue association ontology for 1st level interactors of GLP1RA.** Tissues are sorted by decreasing FDR. On the graph, we highlighted the top 20 tissues for each network. The CV system appeared only for 2nd level interaction networks. The figure was generated from enrichment results in R using ggplot2 and ggrepel libraries. On the left sides of circles are gene numbers and on the right rank based on FDR value.

the GLP1R/GIP network, the genes were; DNAAF3, DNAH5, DNAAF1, NME8, HYDIN, RSPH9, DNAI1, and for both of them; RSPH4A, DNAI2, DNAAF2. We also observed enrichment in this process and genes, as DNAL1, CCNO, SPAG1, OFD1, DNAH8, RPGR for other GLP1RA drugs, but with much higher ranks above 400 to 1773 instead of a rank lower than 10 (Fig. 4D).

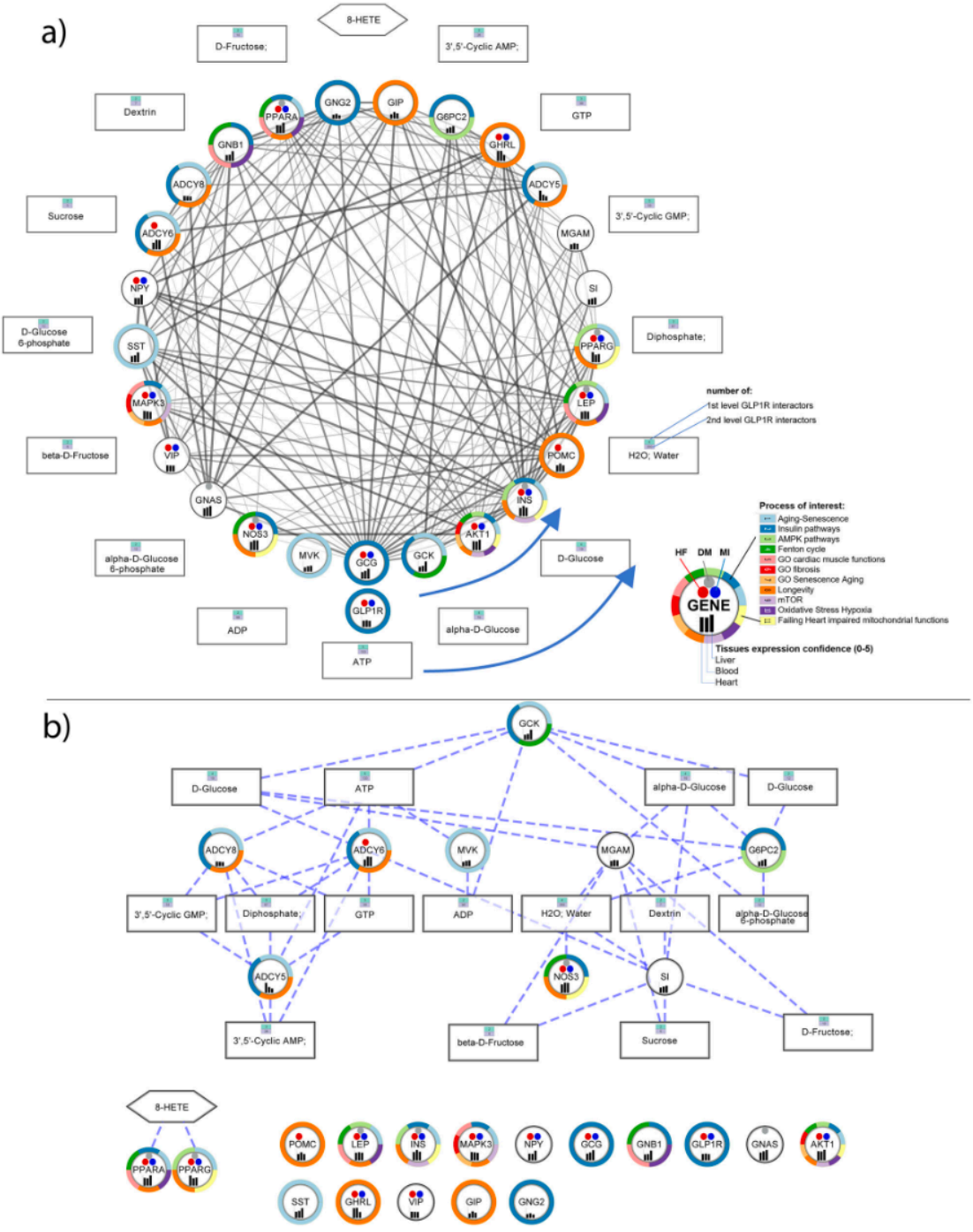
**Verification of specific phenotypes of interest.** The analysis of the term Alzheimer's disease showed its significance for all gene lists, but only for ranks above 5, with the lowest rank being 10 for GLP1R/GIPR/GCGR. In relation to gastroparesis, we observed 4 genes shared among all drugs GHSR, CCK, GHRL, and INS, and 12 genes shared between GLP1R/GIPR/GCGR, GLP1R/GIP, and GLP1R/GIPR: GHSR, REM1, HTR7, PHF20, MLNR, CCK, HTR3A, GHRL, ATP12A, HTR4, MLN, and INS. One specific gene for GLP1R/GIP was HTR4, ranked at 10. The process of gastroparesis was ranked above 5. Pancreatitis enrichment appeared for the primary network with rank 7 ($p$.adj = 1.22E−11) and was ranked 18 for the extended network shared among all gene lists (1.46E−15).

## Metabolites overlap and enrichment analysis

In order to identify which metabolic pathways are regulated by the analyzed drug networks, we performed gene-KEGG ID mapping. We discovered 26 metabolites that overlapped between SGLT2i, metformin, and GLP1RA, and 1446 metabolites shared among all GLP1RA (Fig. 5A, B).

To identify phenotypes, processes, and pathways associated with metabolites related to the analyzed drugs, we performed gene enrichment analysis on the selected metabolite lists. First, we analyzed primary interaction networks, and later, secondary ones (Fig. 5C).

**GLP1R/GIPR/GCGR-related metabolites.** Metabolic pathways specific for GLP1R/GIPR/GCGR were observed for primary and secondary levels of interactions. Primary included short-chain acids and derivatives, TCA acids, citric acid cycle, gluconeogenesis, purine metabolism, pyruvate metabolism, Warburg effect and fructose-1,6-diphosphatase deficiency. Secondary included amines, carbonyl compounds, indolyl carboxylic acids, keto acids and phenyl propylamines (Fig. 5C).




**Overlap between GLP1RA-related metabolites**. We found that metabolites shared between analyzed GLP1RA networks are associated with Galactose metabolism (ATP, ADP, alpha-D-Glucose, D-Fructose, D-Glucose, Glucose 6-phosphate, Pyrophosphate, Sucrose, Water), neomycin, kanamycin and gentamicin biosynthesis, starch and sucrose metabolism, arginine and proline metabolism but also nitric oxide-related pathways, platelet homeostasis (ATP, ADP, citrulline, cyclic GMP, guanosine triphosphate, hydrogen ion, L-arginine, NADPH, nitric oxide, oxygen, pyrophosphate) and VEGF-related pathways (ATP, ADP, citrulline, guanosine triphosphate, hydrogen ion, L-arginine, NADPH, nitric oxide, oxygen). Secondary levels of metabolite-related pathways included acute seizures and other seizure-related disorders, continuous

**Fig. 3 | GLP1R-gene and GLP1R-metabolite interaction networks. a** Top GLP1R 1st level interactors sorted by the degree of connections (inner circle) outside are sorted metabolites associated with those nodes, their interactions are hidden. **b** Interaction network between 1st level GLP1R interactors and top metabolites. Figure was produced in open source Cytoscape software using its native formatting tools degree sorted circle and tree layout. Gray edges depicts gene-gene interaction, blue dotted edges gene-metabolite interaction. Process-of-interest nodes associated with nodes boarder colors (1–11) mark pathway/topic membership via colored node sectors: 1 teal/cyan: Aging/Senescence; 2 sky blue: Insulin pathways; 3 lime/light green: AMPK pathways; 4 green: Fenton cycle; 5 pale red: GO cardiac muscle functions; 6 red: GO fibrosis; 7 light orange: GO senescence/aging; 8 orange: Longevity; 9 lavender/light purple: mTOR; 10 purple: Oxidative stress/Hypoxia; 11 pale yellow: failing-heart-impaired mitochondrial functions; the same colors appear in the network to indicate which processes a gene is linked to. Disease arcs (outer ring) show phenotype associations with three labeled wedges-HF (red-heart failure), DM (gray-diabetes mellitus), and MI (blue-myocardial infarction), a colored arc at a position means the gene is connected to that disease context. Tissue expression confidence (inner bars, 0–5) summarizes evidence of expression in Liver, Blood, and Heart on a 0–5 scale, with taller bars indicating stronger evidence.

ambulatory peritoneal dialysis, biosynthesis of unsaturated fatty acids, steroid biosynthesis and glutamate metabolism (Fig. 5D).

**GLP1R/GIP-related metabolites**. Metabolic pathways specific for GLP1R/GIP appeared only for secondary interactions and included arginine and proline metabolism, D-Arginine and D-ornithine metabolism and benzamides (Fig. 5D).

**GLP1R/GIPR/GCGR related metabolites**. The application of exenatide did not regulate unique metabolites but shared multiple processes with GLP1R/GIPR/GCGR and GLP1R/GIP. The ontological terms shared with the primary network of retatrutide included autism, S-adenosylhomocysteine hydrolase deficiency, cysteine and methionine metabolism, betaine metabolism, disorders of folate metabolism and transport, glyoxylate metabolism, and glycine degradation. There were no significantly enriched terms for the secondary networks (Fig. 5C).

All three drugs shared i.a. arginine and proline metabolism, cardiolipin biosynthesis, de novo triacylglycerol biosynthesis, glutamate metabolism, creatine deficiency, guanidinoacetate methyltransferase deficiency. While for secondary interactions, it was aminoacyl-tRNA biosynthesis, valine, leucine and isoleucine degradation, benzamides 2-Methyl-3-Hydroxybutryl CoA dehydrogenase deficiency (Fig. 5D).

### Comparison of GLP1R agonists with SGLT inhibitors in the context of CVD-related phenotypes

Next, we performed enrichment analysis focused on comparison of GLP1R agonists with SGLT2i in the context of selected CVDs. It revealed stronger enrichment of those phenotypes by GLP1RA than SGLT2i, especially for T2DM and heart diseases (Fig. 6).

### GLP1RA regulation of longevity related genes

In order to evaluate the influence of GLP1RA on longevity-related processes we extracted longevity-related genes interacting with GLP1R. We found 33 genes that are strongly connected and shared between the GLP1R-related networks associated with longevity (Fig. 7). Further analysis revealed that these genes are involved in pathways related to the integration of energy metabolism, adipocytokine pathways, T2D, and the regulation of insulin secretion. Top longevity related genes included INS, AKT1, LEP, PPARG and POMC. Additionally, to add context to GLP1RA targets, we added to the network interactors like GIPR, GCGR, GIP and GCG genes.

### Top GLP1RA interactors

In this part of the study we analyzed the mode of action of various GLP1R/GIPR/GCGR agonists by highlighting their shared and unique direct targets in relation to known receptors. We focused on selected signaling pathways known for being regulated by GLP1RA including cAMP pathway, NF-κB pathway, PI3K pathway, Adipocytokine and adiponectin pathways and other related to interactions with β-arrestin, CAV1, DPP4, ALB, RAMPs and NEP[34–38]. We performed shortest paths analysis in order to identify direct interactors between GLP1RA-related genes and target genes/proteins (Fig. 8). For each pathway we identified top 20 paths connecting them with most direct interactors. Top three interactors for following pathways were identified: cAMP signaling pathway (TSHB, SST, GNAS); NF-κB signaling pathway (TLR4, TNF, RELA); PI3K signaling pathway (GNB5, HSP90AA1 and GNG7). Additionally, we analyzed shortest paths for the AMPK signaling pathway (figure not shown) where the top three targets were G6PC, PPARG and PPARGC1A.

## Discussion

This study aimed for the first time to uncover the connections between genes and metabolites affected by GLP1RA. We used automated data mining from public databases like KEGG, Human Metabolome to analyze gene-related networks for these drugs. This analysis allowed us to identify common processes shared between the drugs as well as specific processes that may represent a potential adverse effect. We also compared our findings with those of SGLT2i and metformin to highlight the similarities and differences between the networks for these drugs.

So far bioinformatic analyses of GLP1RA reveal significant insights into their mechanisms and potential therapeutic applications. Computational modeling has demonstrated that different GLP1RA, such as exendin-F1 and exendin-D3, elicit distinct cAMP signaling dynamics in pancreatic β-cells and neurons, which may influence their efficacy in glucose regulation and appetite suppression[39]. Also, using in silico approaches, pathogenic variants of the GLP1R gene that may impact receptor function and correlate to obesity and diabetes phenotypes have been identified[40]. Meta-analyses have demonstrated that GLP1RA, especially semaglutide, are superior in glycemic control, body weight reduction, and CV events compared to other methods[41,42]. Collectively, these studies underscore the importance of bioinformatics in optimizing GLP1RA therapies for metabolic disorders.

Tissue-specific expression in our analysis highlights the paraventricular nucleus (PVN) and area postrema (AP) as key targets of weight-reducing medications, which impact neural circuits regulating energy balance and eating behaviors. These include hypothalamic centers (PVN, dorsomedial nucleus [DMN], arcuate nucleus [ARC]), dopaminergic pathways (nucleus accumbens [NAc], substantia nigra [SNc], ventral tegmental area [VTA]), and brainstem nuclei (parabrachial nucleus [PBN], nucleus of the solitary tract [NTS], DMV, AP)[43].

The PVN modulates appetite and energy expenditure via hormones like CRH and TRH, while the ARC, with its appetite-stimulating (NPY, AgRP) and satiety-promoting (POMC) neurons, regulates feeding behaviors[44,45]. Dopaminergic pathways (NAc, SNc, VTA) influence reward-driven eating, aiding adherence to dietary changes[46,47]. Brainstem nuclei integrate sensory and autonomic signals, enhancing medication effects on energy balance[44,45,47]. These findings emphasise the complex neural networks targeted by weight-loss drugs, supporting appetite suppression, satiety, and reduced reward from eating, vital for effective interventions.

GLP1RA, while generally well-tolerated, can be associated with some side effects. It's important to note that individual responses can vary, and not every patient will experience these effects. Common side effects of GLP1RA may include: Gastrointestinal Distress: The most frequently reported side effects are related to the gastrointestinal system. This can include nausea, vomiting, diarrhea, and abdominal pain. These symptoms are often more pronounced when treatment is initiated and may decrease over time. In some cases, gastroparesis is characterized by delayed gastric emptying in the absence of mechanical obstruction. Studies indicate that the gastrointestinal deceleration effects observed in liraglutide (Victoza/Saxenda)[21] and

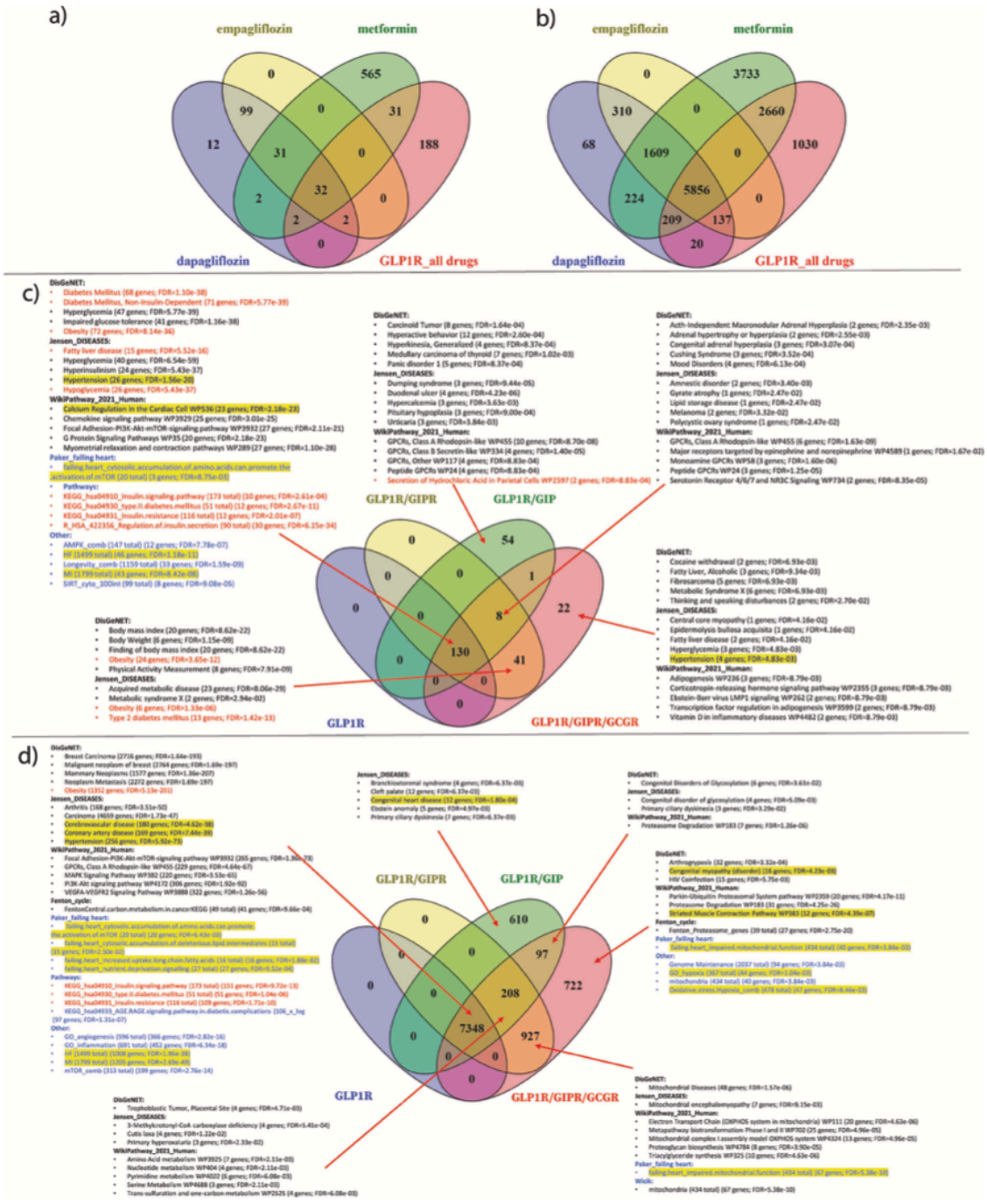



**Fig. 4 | Venn diagrams of genes overlap for GLP1R-related drugs. a** 1st level interactors and **b** both levels interactors of SGLT2i, metformin and GLP1RA. Venn diagrams and top 5 enriched terms for; **c** 1st level interactors and **d** both levels of interactors of GLP1RA. Enrichment cut-off was set for ontological terms with at least 2 enriched genes and FDR adjusted $p$ value ≤0.05. Redundant terms were removed. Venn diagrams were created using open source Venny 2.1 web. Yellow filled labels depict heart-related processes, red label-recognized effects, and blue label custom pathways from Fisher exact test.

semaglutide (Ozempic/Wegovy)[48] are primarily transient. However, there is a suggestion that such effects might endure for a more prolonged duration in the case of the shorter-acting twice-daily exenatide (Byetta)[49]. In our study, we observed a significant impact on hydrochloric acid secretion. Regarding other side effects, while GLP1RA themselves are not typically associated with hypoglycemia, when used in combination with other antidiabetic medications, there may be an increased risk of hypoglycemia. This is especially relevant when combined with insulin or sulfonylureas.

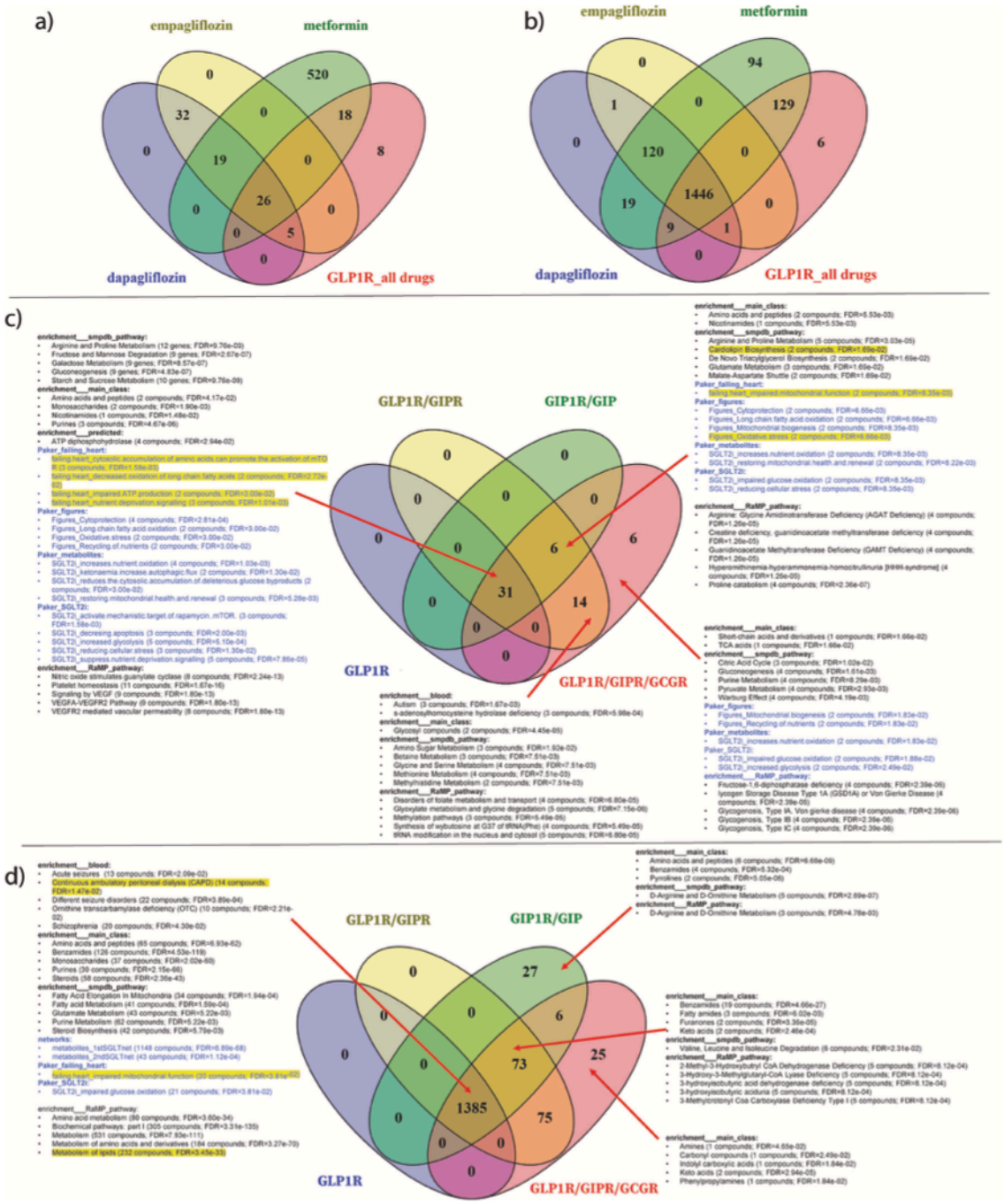



**Fig. 5 | Venn diagrams of metabolites overlap for GLP1R-related drugs. a** 1st level interactors and **b** both levels of interactors of SGLT2i, metformin and GLP1RA. Venn diagrams and top 5 enriched terms for **c** 1st level interactors and **d** both levels of interactors of GLP1RA. Enrichment cut-off was set for ontological terms with at least 2 enriched metabolites and FDR adjusted $p$ value ≤0.05. Redundant terms were removed. Venn diagrams were created using open source Venny 2.1 web app. Yellow filled labels depict heart-related processes, red label-recognized effects, and blue label custom pathways from Fisher exact test.

Hypoglycemia was strongly represented in our study. Since most GLP1RA are administered by injection, mild reactions at the injection site may occur, such as redness or itching. There have been reports of pancreatitis associated with the use of GLP1RA. While the incidence is low, individuals with a history of pancreatitis may need careful consideration before using these medications. There has been some evidence suggesting an increased risk of thyroid C-cell tumors in rodents with long-term use of GLP1RA[50]. However, the relevance of this finding to humans is not yet fully understood.

Despite observing expected processes associated with GLP1R activity, like influence on T2D, hyper- and hypoglycaemia and obesity we observed a

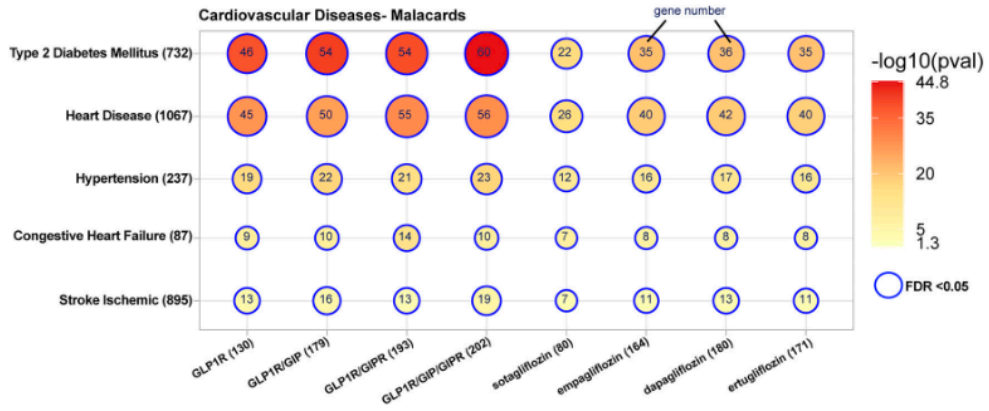
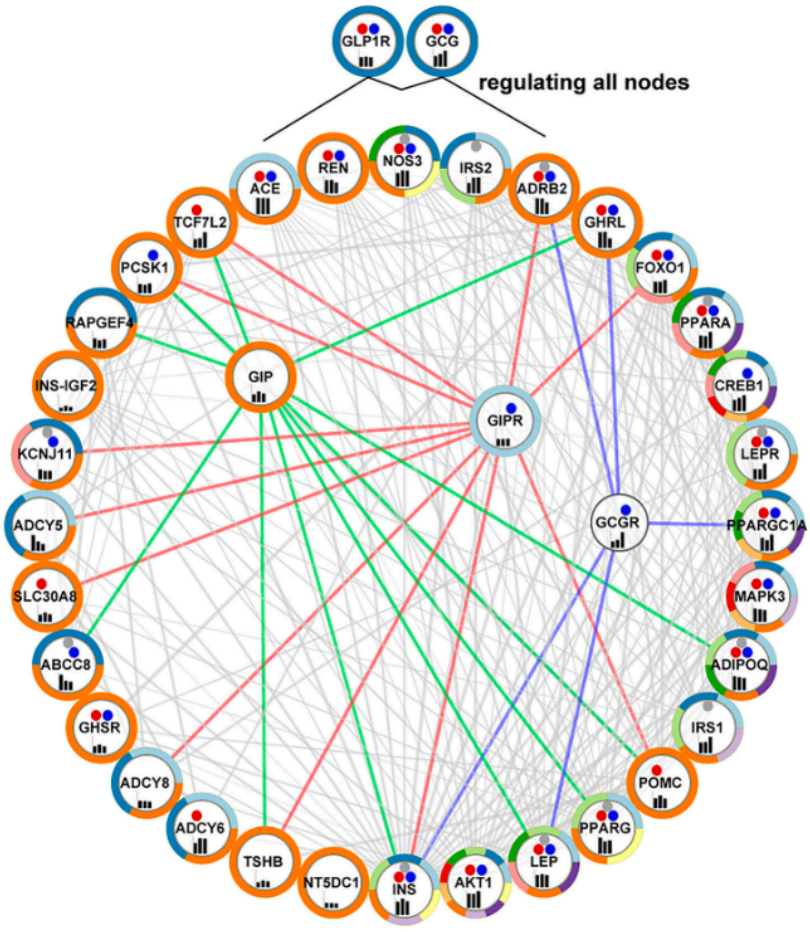



**Fig. 6 | Enrichment analysis of GLP1RA and SGLT2i in context of selected CVD related phenotypes.** Node size is associated with enriched gene numbers. Analysis was performed for first level interaction networks of selected drug targets. The figure was generated from enrichment results in R using ggplot2 and ggrepel libraries.

**Fig. 7 | Visualization of an interaction network formed by longevity related genes regulated by GLP1R.** Genes were sorted by the decreasing number of connections. Figure was produced in Cytoscape software using its native formatting tools and degree sorted circle layout. Process-of-interest nodes associated with nodes boarder colors (1–11) mark pathway/topic membership via colored node sectors: 1 teal/cyan: Aging/Senescence; 2 sky blue: Insulin pathways; 3 lime/light green: AMPK pathways; 4 green: Fenton cycle; 5 pale red: GO cardiac muscle functions; 6 red: GO fibrosis; 7 light orange: GO senescence/aging; 8 orange: Longevity; 9 lavender/light purple: mTOR; 10 purple: Oxidative stress/Hypoxia; 11 pale yellow: failing-heart-impaired mitochondrial functions; the same colors appear in the network to indicate which processes a gene is linked to. Disease arcs (outer ring) show phenotype associations with three labeled wedges-HF (red-heart failure), DM (gray-diabetes mellitus), and MI (blue-myocardial infarction); a colored arc at a position means the gene is connected to that disease context. Tissue expression confidence (inner bars, 0–5) summarizes evidence of expression in Liver, Blood, and Heart on a 0–5 scale, with taller bars indicating stronger evidence.




plethora of other processes and pathways which could be considered a side effect of GLP1R targeted therapy.

Gene overlap analysis and GLP1R/GIPR/GCGR network-specific genes showed an enrichment of fatty liver disease. We also observed an enrichment of alcoholic fatty liver disease for the GLP1R/GIP network. GLP1RA, beyond lowering blood sugar and providing heart and kidney benefits, significantly influence weight management and improve clinical, biochemical, and histological markers of fatty liver and fibrosis in metabolic-

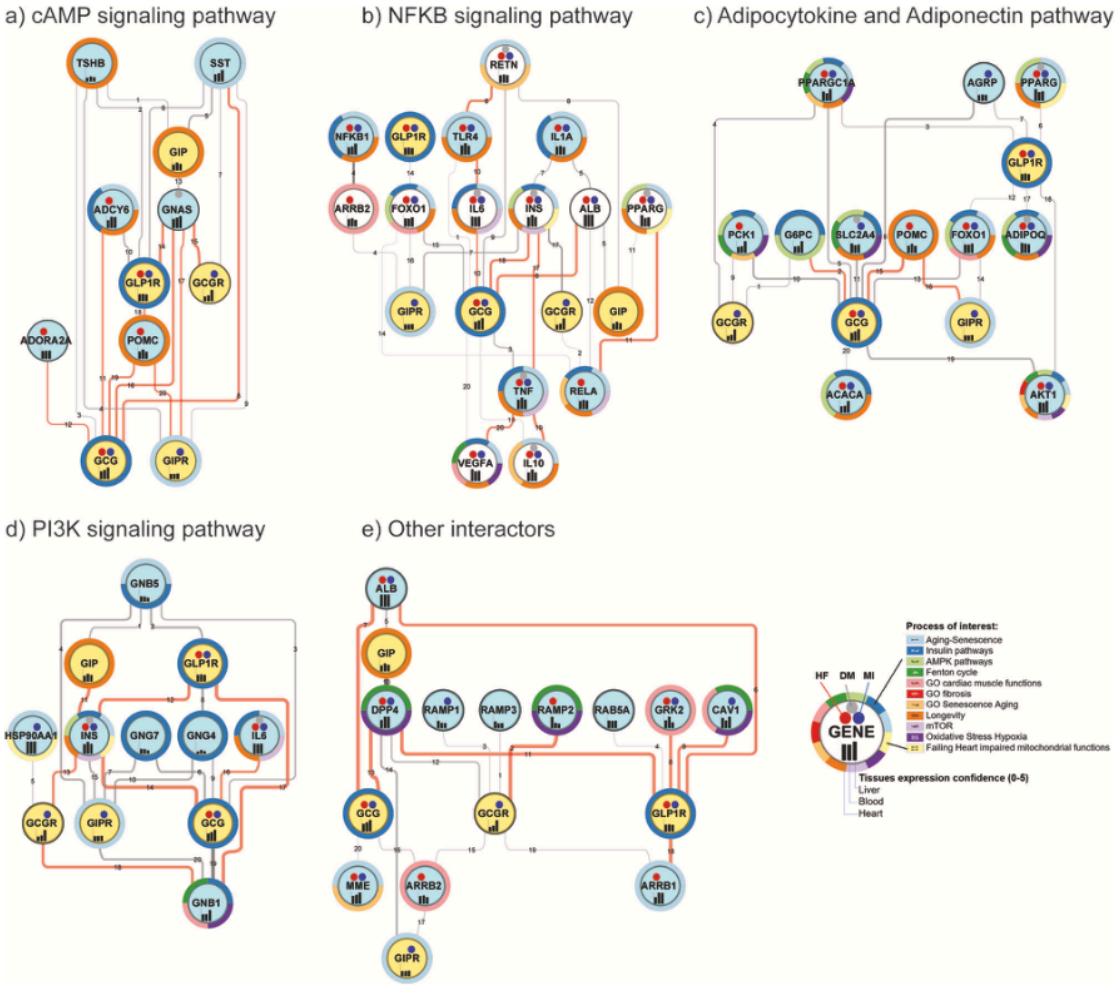



**Fig. 8 | Shortest paths analysis between various GLP1RA-related genes.** GLP1RA-related genes (yellow fill) and their top direct interactors (light blue fill). For each network we selected top 20 pathways: **a** cAMP signaling pathway, **b** NFKB signaling pathway, **c** Adipocytokine and Adiponectin pathway, **d** PI3K signaling pathway, **e** other incretin-related interactors. Paths marked with red have high confidence String database score (>0.7). Numbers on the edges significant number of shortest path. Figure was produced in Cytoscape software using its native formatting tools and hierarchical layout. Process-of-interest associated with nodes boarder colors (1–11) mark pathway/topic membership via colored node sectors: 1 teal/cyan: Aging/Senescence; 2 sky blue: Insulin pathways; 3 lime/light green: AMPK pathways; 4 green: Fenton cycle; 5 pale red: GO cardiac muscle functions; 6 red: GO fibrosis; 7 light orange: GO senescence/aging; 8 orange: Longevity; 9 lavender/light purple: mTOR; 10 purple: Oxidative stress/Hypoxia; 11 pale yellow: failing-heart-impaired mitochondrial functions; the same colors appear in the network to indicate which processes a gene is linked to. Disease arcs (outer ring) show phenotype associations with three labeled wedges-HF (red-heart failure), DM (gray-diabetes mellitus), and MI (blue-myocardial infarction); a colored arc at a position means the gene is connected to that disease context. Tissue expression confidence (inner bars, 0–5) summarizes evidence of expression in Liver, Blood, and Heart on a 0–5 scale, with taller bars indicating stronger evidence.

associated fatty liver disease (MAFDL)[51]. While weight loss of 7–10% remains the only proven intervention for improving fibrosis and steatosis in MAFDL, evidence on GLP1RA' effectiveness in resolving pre-existing liver fibrosis is lacking. Our findings indicate strong potential for GLP1R drugs to impact this process positively, with enhanced effects observed for GLP1R/GIPR/GCGR on fatty liver, consistent with reports that 70% of T2D patients have fatty liver. In a study of 98 obese adults, semaglutide reduced liver fat by 81.7% (8 mg) and 86% (12 mg) at 48 weeks[52].

Our analysis revealed enrichment of 26 genes related to hypertension. GLP1RA improve blood pressure and reduce CV event risk, likely through central and peripheral autonomic regulation, increased natriuresis, and the presence of these genes in CV tissues (e.g., endothelial cells, vascular smooth muscle cells, resistance arteries)[53]. Research suggests GLP1RA reduces vascular inflammation via endothelial GLP1R, not myeloid GLP1R[54]. Our study highlights GLP1R's direct influence on hypertension-related genes in pathways such as adipocytokine signaling, fructose metabolism, CRP, and inflammation, alongside potential effects on calcium metabolism and mTOR activation. Top interactors linked to MI and HF include GLP1R, GCG, AKT1, INS, POMC, LEP, PPARG, GHRL, PPARA, ADCY6, NPY, MAPK3, VIP, and NOS3.

Our study identified hyperactive behavior and panic disorder as potential side effects of GLP1R/GIP agonists, with mood disorders impacted across all drug networks. Genes related to cocaine withdrawal were enriched in the GLP1R/GIPR/GCGR network, aligning with evidence that GLP1RA may target psychostimulant abuse[55,56]. Activation of central GLP1Rs reduces anxiety and depression behaviors in rodents[57,58], while intravenous GLP-1

lacks anxiogenic effects in humans, even in panic disorder patients[59–61]. A meta-analysis found GLP1RAs exhibit antidepressant effects, particularly in T2D, with liraglutide showing notable efficacy over exenatide, though subgroup differences were not significant[62]. Additionally, we observed enrichment of 22 genes related to seizure disorders, supported by evidence that GLP1R may suppress seizures through neuronal receptor regulation[63].

In our study, we observed an overrepresentation of terms related to the GLP1R/GIPR/GCGR network such as the Parkin-Ubiquitin Proteasomal System pathway and Proteasome degradation. Data from animal models and preclinical studies indicate that GLP1RA may help restore dopamine levels, prevent dopaminergic loss, reduce neuronal degeneration, and alleviate both motor and non-motor symptoms of Parkinson's disease. Clinical studies also show promising results, suggesting that GLP1RA could be a valuable addition to the current array of drugs available for Parkinson's disease treatment[20].

In our study, we observed an increase in duodenal ulcer-related terms for the GLP1R/GIP network. A study by Inagaki et al. in 2022 found that tirzepatide did not cause duodenal ulcers in any participants, unlike dulaglutide[64]. Recent literature suggests that tirzepatide, may cause or worsen duodenal ulcers in susceptible individuals. This effect is thought to result from changes in gastric acid secretion, gastric emptying, and gastrointestinal motility mediated by GIP/GLP-1 activation. Clinical trials have reported a higher incidence of gastrointestinal adverse events, including duodenal ulceration, compared to other antidiabetic agents[65].

Our findings suggest a potential impact on calcium levels and pituitary hyperplasia in the GLP1R/GIP network. When studying GLP1R knockout mice, it was found that there were no changes in serum calcium or parathyroid hormone concentrations[66]. However, there was no observation of cortical bone osteopenia, bone fragility, increased bone resorption, and reduced calcitonin mRNA in the thyroid gland[67]. According to existing literature, it appears that the GLP1R in C-cells, along with its interaction with calcitonin, plays a vital role in regulating bone resorption and mass in mice[67]. Additionally, when GLP1R knockout mice were used to validate the impact of exenatide and liraglutide, no increase in calcitonin secretion or C-cell hyperplasia was observed. Moreover, clinical data from 17 patients suggested that early monitoring of TSH after starting tirzepatide might be necessary to avoid iatrogenic hyperthyroidism in patients with hypothyroidism[68]. Our results align with these findings, highlighting the importance of such monitoring to prevent potential complications. Additionally, in our shortest paths analysis we identified TSHB as a potential key player in cAMP pathway.

Furthermore, we noted an increased presence of carcinoid tumors and medullary carcinoma of the thyroid in the context of the GLP1R/GIP network. This aligns with existing literature, which indicates that exposure to a GLP1RA for 1–3 years significantly raised the risk of all thyroid carcinomas by 58% (hazard ratio: 1.58; 95% confidence interval: 1.27–1.95) and medullary thyroid cancer by 78% (hazard ratio: 1.78; 95% confidence interval: 1.04–3.05)[50]. Given these findings, close observation of this issue is particularly warranted.

Our findings indicated that the GLP1R/GIP networks have an impact on hydrochloric acid secretion and primary ciliary dyskinesia. We also observed this effect with GLP1R/GIPR/GCGR and GLP1R/GIPR networks, not just with GLP1R alone. Primary ciliary dyskinesia (PCD) is a rare autosomal recessive genetic disorder caused by malfunctioning motile cilia, primarily affecting the respiratory system. Studies link PCD to an increased risk of conditions like rheumatoid arthritis, congenital heart disease, severe esophageal diseases, and possibly type 1 diabetes (T1D). Notably, GLP1RA trials have reported infections such as upper respiratory, urinary tract, nasopharyngitis, influenza, cystitis, and viral infections[69]. Our study can help explain the source of these side effects.

Regarding gastroparesis we observed 4 genes shared between all drug networks on the first level network GHSR, CCK, GHRL, INS. Twelve genes were shared between all drugs on the second level network GHSR, REM1, HTR7, PHF20, MLNR, CCK, HTR3A, GHRL, ATP12A, HTR4, MLN, INS. One was specific for GLP1R/GIP HTR4. Semaglutide use is increasingly associated with gastroparesis, a condition marked by delayed gastric emptying, causing nausea, vomiting, abdominal pain, bloating, satiety, acid reflux, and blood sugar fluctuations. Severe cases often result from vagus nerve damage, which controls stomach muscles. Studies show GLP1RA for weight loss, compared to bupropion-naltrexone, are linked to higher risks of pancreatitis, gastroparesis, and bowel obstruction, but not biliary disease[70].

The drugs known as GLP1RAs have shown potential benefits for various age-related conditions and complications, such as chronic kidney disease (CKD) and Alzheimer's disease[71–73]. It's not surprising considering that individuals diagnosed with T2D at age 30 could see a reduction in life expectancy by as much as 14 years[74]. In our study, we observed a significant overrepresentation of longevity-related ontological terms and related processes. For example, we observed multiple changes in mitochondrial activity levels, particularly for the GLP1R/GIPR/GCGR extended interaction network associated with HF, mitochondrial diseases, and the combined list of all known mitochondrial genes. Retatrutide, which activates these receptors, appears to alleviate oxidative stress in liver mitochondria, a significant factor in the progression of MASLD. Enhancing fat oxidation has the potential to improve mitochondrial function. Literature data confirm these findings, showing that GLP1RA improved the redox state and mitochondrial respiration of leukocytes, and diminished leukocyte-endothelial interactions, inflammation, and carotid intima-media thickness[75]. Other results also suggest that GLP-1 increases ER-mitochondria communication in vascular smooth muscle cells, resulting in higher mitochondrial activity[76]. Our visualization of the interaction network formed by longevity-related genes regulated by GLP1R (Fig. 7). With top factors identified as INS, AKT1, LEP and PPARG. Those factors were also present in the interaction network for GLP1R, pointing out the close association of the influence of GLP1RA on longevity-related processes.

The top interactor in our metabolite network and gene-gene network was glucokinase gene GCK. GCK acts as a glucose sensor in pancreatic beta cells, influencing insulin secretion and with rising blood glucose, it increases the quantity of insulin produced. Research indicates that GCK activity can enhance the effectiveness of GLP1RA by promoting insulin release in response to elevated glucose levels, thereby improving glycemic control[77]. GCK plays a major role in recognizing how high the blood glucose level is within the body, which means when glucokinase is working normally, blood glucose will not go very high. Glucokinase diabetes is one of the familial diabetes types that together are often called MODY (maturity onset diabetes of the young)[78]. GCK appears to regulate GLP1R activity by modulating GRK involvement and influencing receptor conformational states during activation[79]. Animal models suggest that while β-cell glucokinase is crucial for liraglutide-induced insulin secretion, liraglutide may still enhance glycemic control, reduce steatosis, and prevent β-cell death through mechanisms independent of glucokinase[80].

Top 2 metabolites affected by the GLP1R were ATP and alpha-D-Glucose. Upon GLP-1 binding to its receptor GLP1R on pancreatic beta cells, a signaling cascade is initiated, leading to the activation of adenylyl cyclase and the production of cAMP), which activates protein kinase A (PKA) enhancing insulin gene transcription and insulin secretion[81]. Alpha-D-Glucose stimulates GLP-1 secretion from intestinal L-cells, activating GLP1R on pancreatic beta cells to enhance insulin secretion[44]. The top lipid metabolite 8-HETE (8-Hydroxyeicosatetraenoic Acid), an oxidized lipid mediator, modulates GLP1R sensitivity and signaling, influencing metabolic responses and insulin secretion dynamics[82].

In a simulated metabolites analysis, several common processes relevant to HF were identified, aligning with the results from gene network analyses. Interestingly, among the identified changes, there were significant observations regarding platelet homeostasis. Platelets play a crucial role in maintaining the balance of blood clotting and are involved in CV health and disease progression. The enrichment of platelet homeostasis pathways suggests a potential link between metabolic dysregulation and platelet function in the context of HF, highlighting the importance of understanding platelet biology in CV pathophysiology.

Additionally, our study revealed the relationship between GLP1R and platelet homeostasis. This has received little attention in the literature[83,84]. The connection between GLP1R signaling and platelet function requires further investigation, especially considering the potential therapeutic implications considering that platelets are important actors of CVD.

Besides GCG gene and GCK we identified AKT1 as the top interactor in the GLP1RA network. Variations in AKT1 have been linked to schizophrenia, bipolar disorder, T2D and Parkinson's disease[85]. Identification of this gene as top interactor can explain the potential side effects of GLP1RA in our study, like alterations in behavior, enrichment of Parkinson's disease. Additionally, GLP1R activation exerts an antitumor effect on human pancreatic cancers by inhibiting the PI3K/Akt pathway. This suggests that GLP-1-based therapies could be beneficial, rather than harmful, for treating patients with T2D with pancreatic cancer[86].

To explore mechanisms beyond canonical receptor binding, we examined GLP1R-related genes and receptors in the context of signaling pathways known to be influenced by incretin therapy. Using a shortest-paths analysis, we identified direct interactors within the cAMP, NF-κB, AMPK, and adipocytokine/adiponectin pathways, along with proteins previously reported to modulate GLP1R function (β-arrestin, CAV1, DPP4, RAMPs, NEP). In the cAMP pathway, top interactors included GNAS, SST, and TSHB. GNAS. GNAS gene is an important regulator of insulin secretory capacity in pancreatic β-cells and when it is activated, it stimulates enzyme adenylate cyclase to produce cAMP[87]. Regulation of SST and TSHB suggests broader endocrine connections. In the NF-κB pathway, TNF, RELA, and TLR4 were prominent, supporting reports that GLP1RAs suppress inflammatory signaling and modulate innate immune responses[88]. For the AMPK and adipocytokine/adiponectin pathways, recurrent nodes included G6PC, PPARG, and PPARGC1A, genes central to hepatic glucose regulation, adipose metabolism, and mitochondrial function, pathways consistently linked to GLP1RA metabolic benefits. In the PI3K pathway, GNB5, HSP90AA1, and GNG7 were identified, highlighting G-protein subunits and chaperones known to influence Akt signaling. GNB5 may play an important role in neuronal signaling, including in the parasympathetic, but not sympathetic, control of heart rate[89]. Overall, our results confirm the involvement of well-established mediators (e.g., GNAS, RELA, PPARG) while pointing to less-characterized interactors (e.g., TSHB, GNB5) that may contribute to the pleiotropic effects of incretin-based therapies.

This study has several limitations. First, it is based solely on in silico analyses, and the predicted interactions require experimental validation. Second, the results depend on the completeness of available databases, which may introduce bias or omit relevant interactions. Future experimental and clinical studies will be essential to validate and extend these systems-level insights.

Taken together, both GLP1RA and SGLT2i are well-established classes of drugs for managing T2D, recognized for their CV benefits. Our in silico analysis demonstrated that GLP1R, either alone or in combination with GLP1R/GIP/GIPR, shows a stronger association with genes targeting T2D, HF, hypertension, and congestive HF compared to SGLT2i. Besides characterization of GLP1RA interaction networks in our study, we conducted an enrichment analysis to compare the effects of GLP1RA with SGLT2i in the context of various CVDs. The results indicated a more pronounced enrichment of certain phenotypes associated with GLP1RA compared to SGLT2i, highlighting their potential advantages, particularly in relation to T2D and heart diseases. This finding suggests that GLP1RA may offer greater therapeutic benefits in these conditions, underscoring their importance in the management of CVDs linked to metabolic disorders.

## Methods

### Construction of the interaction networks for drug targets

In order to identify first and second level interactors for each gene/drug combination GLP1R (semaglutide), GLP1R/GIPR (tirzepatide), GLP1R/GIPR/GCGR (retatrutide) and additionally GLP1R/GIPR/GIP, we used gene symbols as seeds. For empagliflozin, dapagliflozin, ertugliflozin, metformin we obtained targeted genes from BindingDB. Human interactome version 12.0 was obtained through stringApp v2.11[90] using Cytoscape software v3.9.1[91] and imported to R. From R level we extracted first-level = direct neighbors of seed targets; second-level = all neighbors of first-level nodes. We used as evidence channel stringdb score 0.4 which is medium confidence. We selected this cut-off based on preliminary analyses and our previous studies. Results were saved as gene lists and used for further enrichment analyses.

The interaction network for GLP1R was constructed in Cytoscape using String App using the same parameters and further integrated with gene-metabolites interactions constructed in R. Longevity related network was also constructed in Cytoscape based on shared longevity-related genes from GLP1R network. Interaction networks from figures are available in Supplementary data 2. Gene lists of complete first and second level networks are provided in Supplementary data 1 in spreadsheet "networks_genes". Legend for network figures and content description of Supplementary data 2 is in Supplementary File 1.

### Selection of gene and metabolite lists

In order to more precisely identify processes in gene set enrichment analyses by using bioinformatic tools we aimed to identify genes and metabolites associated with processes which would be relevant to action of GLP1RA and SGLT2i in the context of CV health. Several gene sets related to HF were retrieved based on Milton Packer publication[92]. In line with the framework proposed by Packer, the gene sets could be broadly classified into three mechanistic domains. Failing heart-relevant processes included increased uptake of long-chain fatty acids, impaired ATP production, cytosolic accumulation of deleterious lipid intermediates, impaired mitochondrial function, suppression of nutrient-deprivation signaling, cytosolic accumulation of amino acids driving aberrant mTOR activation, and inflammation-mediated functional iron deficiency with altered metabolism. By contrast, SGLT2i-relevant processes encompassed increased glycolysis, impaired glucose oxidation, reduction of cellular stress, attenuation of pro-inflammatory signaling, decreased apoptosis, limitation of cytosolic accumulation of deleterious glucose intermediates, modulation of mTOR signaling, suppression of nutrient-deprivation pathways, restoration of mitochondrial health and renewal, enhanced nutrient oxidation and oxidative phosphorylation, reduced accumulation of toxic glucose and lipid by-products, stimulation of ketonaemia, induction of ketone-driven autophagic flux, and regulation of iron handling through increases in cytosolic $Fe^{2+}$ accompanied by reductions in ferritin and hepcidin levels. Finally, a third group reflected SGLT2i influences on metabolites, comprising reversal of abnormalities in long-chain fatty acid uptake, provision of ketonaemia as an alternative fuel that augments autophagic flux, improvement in iron homeostasis (increasing the bioreactive $Fe^{2+}$ pool while lowering ferritin and hepcidin), and mitigation of cytosolic accumulation of harmful glucose and lipid by-products, accompanied by an overall enhancement of mitochondrial oxidative metabolism. Specific gene and metabolites lists were retrieved directly from publication text and figures and also obtained using these specific key terms from ref. 93, with curated use of artificial intelligence, KEGG (https://www.kegg.jp/) and GeneOntology (https://geneontology.org/) databases. All these gene sets are listed in Supplementary data 1.

Genes related to the Fenton reaction included those associated with the Warburg effect and proliferation, cytosolic iron-sulfur cluster synthesis, neutralization of $H_2O_2$, NADPH-dependent dehydrogenases, exogenous superoxide synthesis, cytosolic iron transporters, the proteasome, the oxidative-to-glycolytic shift, the Warburg effect, and central carbon metabolism in cancer obtained from KEGG database (https://www.kegg.jp/).

Other gene lists were custom-designed to capture broader biological processes and disease-relevant pathways. These included sets related to oxidative stress and hypoxia, aging and senescence (GO database, https://geneontology.org/), AMPK signaling (Reactome), mTOR signaling (Reactome), longevity pathways (https://www.kegg.jp/[94]), Genome maintenance (GO database), mitochondrial function (GO database). Additional custom lists comprised sirtuin genes and sirtuin interactors (selected in Cytoscape), as well as disease-related categories such as myocardial infarction (MI) and

HF obtained from DisGenet database (https://disgenet.com/). Functionally enriched sets were also designed from Gene Ontology terms, including apoptosis, coagulation, platelet activation, inflammation, hematopoiesis, angiogenesis, cardiac muscle functions, muscle hypertrophy, fibrosis, oxidative stress, senescence and aging, and hypoxia which we used in our previous studies relevant to cardiovascular disease (CVD)[95,96]. For Fisher exact test comparing CVD phenotypes between GLP1RA and SGLT2i we extracted related gene lists from Malacards database (https://www.malacards.org/): MI, HF, Ischemic stroke, Heart disease, Hypertension, Congestive HF, T2DM. Fisher's exact test was performed against all genes in the human genome. Complete list of genes and metabolites associated with analyzed processes is in the Supplementary data 1.

### Identification of compounds (metabolites) associated with the networks

First we translated Entrez IDs to hsa gene ids in the KEGG database using the keggConv() function from the KEGGREST package. Further we performed annotation of genes to compounds using the same package and functions keggGet(). Compounds were mapped using the website-based REST system according to template (example: link_code<-paste("https://www.genome.jp/dbget-bin/get_linkdb?-t+compound+",geneID,sep="")). Additional annotations for compounds were also obtained using keggGet() and REST based screening of metabolomicsworkbench.org to obtain HMDB ids. This allowed merging the metabolites with the nodes from the networks.

### Annotation methods

For mapping genes to the KEGG database- we used the KEGGREST package and function keggGet(). For mapping genes to pathways from the KEGG database- we used keggList() function. For mapping genes to KEGG compounds (metabolites)- we used KEGG REST website.

HMDB IDs were further mapped to names of metabolites using our R function metabolites_to_genes() performing annotation of metabolites and identification of genes associated with the metabolites through HMDB database[97]. This allowed merging the metabolites with the nodes from the network. KEGG Compound IDs were mapped using KEGGREST API and our function keggGenes_compound(). Before we annotated the genes to KEGG IDs and pathways using our function KEGGgene_mapping().

### Enrichment analysis

Enrichment analysis of genes was done using the API for EnrichR database (https://maayanlab.cloud/Enrichr/) by implementing Hypergeometric test with Benjamini and Hochberg correction, while the reference was the human genome. For enrichment analysis the following databases were used: Descartes_Cell_Types_and_Tissue_2021, DisGeNET, Jensen_DISEASES, Jensen_TISSUES, WikiPathway_2021_Human[98].

Custom gene lists and tissues (Tissues 2.0 database) were analyzed using Fisher Exact test with false discovery rate (FDR) correction ($p < 0.05$). For tissue enrichment analysis we downloaded JENSEN tissues database (https://download.jensenlab.org/human_tissue_integrated_full.tsv), calculated mean expression of confidence for each tissue and selected for enrichment analysis the top 30% of high-confidence genes[99]. For all statistical analyses, the significance cut-off was set to an adjusted $p$-value ≤ 0.05.

Metabolites (KEGG compounds) enrichment analysis was done using MetaboAnalyst enrichment analysis tool (https://new.metaboanalyst.ca/MetaboAnalyst/upload/EnrichUploadView.xhtml). For data aggregation and ranking, we used our wizbionet R package[100]. Results of this enrichment analysis are in Supplementary data 1. Enrichment analysis of Tissues and CVDs was performed using Fisher's exact tests on the Tissues 2.0 database (we selected the top 30% genes with the highest confidence of expression) and gene sets obtained from MeSH (https://www.malacards.org/). Visualization of ontological results of tissue association ontology and CVD analysis was performed in R using ggplot2 and ggrepel libraries.

### Shortest paths analysis

Shortest-path analyses were conducted using the PathLinker Cytoscape App[101] with the following parameters: an unweighted, undirected network and the target set (GLP1R/GCGR/GIPR/GCG/GIP), and sources defined as genes linked to signaling pathways from curated databases. Path linker identifies shortest paths between source and target nodes in the interaction network, in this case, the whole interactome. It reconstructed signaling or functional pathways by computing the k-shortest paths, using a user-defined target protein and a set of source genes. The resulting ranked paths highlight likely molecular routes connecting sources to the target within the network.

Source pathways were: cAMP signaling pathway (https://reactome.org/content/detail/R-HSA-170660, https://www.kegg.jp/entry/ko04024); NF-κB signaling pathway (https://www.kegg.jp/entry/ko04064, https://www.gsea-msigdb.org/gsea/msigdb/human/geneset/BIOCARTA_NFKB_PATHWAY.html); AMPK pathway[95], PI3K pathway (https://www.kegg.jp/entry/ko04151, https://reactome.org/content/detail/R-HSA-198203), Adipocytokine and adiponectin pathway (https://www.creative-diagnostics.com/adiponectin-signaling-pathway.htm, https://www.kegg.jp/pathway/map04920+K07296), β-arrestin related genes (Gene Ontology), Caveolin 1 (CAV1), Dipeptidyl Peptidase 4 (DPP4), albumin (ALB), RAMPs and neprilysin (NEP). From the results, we examined the top 20 paths associated with each key term.

## Data availability

The main datasets analyzed during the current study are available in Supplementary data 1. Cytoscape networks are available in Supplementary data 2.

## Code availability

The underlying code for this study is not publicly available but may be made available to qualified researchers on reasonable request from the corresponding author.

Received: 30 June 2025; Accepted: 2 November 2025;
Published online: 23 November 2025

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## Acknowledgements

This work was supported by the Medical Research Agency of Poland (ABM), grant no. 2019/ABM/01/00037, “Empagliflozin and dapagliflozin in patients hospitalized with acute heart failure: a phase III study (EMPATHY)”. This work was also supported by the grant no. 2020/37/B/NZ2/03757 "Multiscale spatial reorganization of chromatin in response to replication stress and its role in cellular protection against genomic instability".

## Author contributions

Z.W., M.P. developed the study concept and design, Z.W., A.N.S. collected and assembled the data, Z.W. performed data analysis and visualization, Z.W., M.P., C.E. data interpretation, Z.W., A.N.S., C.E., J.B. wrote the manuscript; manuscript supervision: S.H., S.K., D.v.L., M.P., and all authors read, reviewed, and approved the final version of the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41540-025-00619-6.

**Correspondence** and requests for materials should be addressed to Marek Postuła.

## 6.3. MicroRNAs and long non-coding RNAs associated with sirtuin pathways in ischemia/reperfusion injury

Cardiovascular Drugs and Therapy
https://doi.org/10.1007/s10557-026-07860-z

REVIEW

# MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury

Anna Nowak-Szwed[1,2,3] · Ceren Eyileten[1,4,8] · Zofia Wicik[1,5] · Sara Ahmadova[1,3] · Dirk von Lewinski[6] · Harald Sourij[7] · Marek Postula[1]

Received: 3 November 2025 / Accepted: 18 February 2026


**Abstract**
Myocardial ischemia/reperfusion (I/R) injury contributes significantly to post-infarction cardiac dysfunction and heart failure, despite advances in reperfusion therapies. Among molecular regulators of I/R injury, sirtuins (SIRTs) play key roles in modulating oxidative stress, apoptosis, inflammation, and mitochondrial function. Increasing evidence highlights the regulatory role of non-coding RNAs (ncRNAs), including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), in controlling sirtuin signaling in the ischemic myocardium. This review synthesizes current preclinical findings on miRNA- and lncRNA-mediated regulation of SIRTs in myocardial I/R injury model. It further highlights the emerging mechanistic pathways through which ncRNAs influence sirtuin activity and discusses their potential relevance as therapeutic targets. Several miRNAs aggravate cardiomyocyte damage by downregulating SIRTs, while lncRNAs exert protective effects through miRNA sponging and sirtuin upregulation. These regulatory axes influence key cellular processes, including mitochondrial homeostasis, pyroptosis, apoptotic signaling and regulation of the inflammasome pathways. Additionally, the network analysis identified apoptosis as the most frequently involved process, with SIRT1 and miR-29a, miR-34a, and miR-217-5p showing the highest degree of connectivity. Despite growing mechanistic insight, translation into clinical practice is hindered by the scarcity of human studies and randomized trials. Moreover, current knowledge regarding miRNAs is limited to only three sirtuin isoforms, underscoring the need for further investigation. Understanding the ncRNA–SIRT axis may offer novel therapeutic strategies for mitigating myocardial I/R injury.

**Keywords** microRNA · miRNA · lncRNA · ncRNA · Sirtuin · Ischemia/reperfusion injury · Myocardial infarction

## Introduction

Myocardial infarction (MI) used to be considered one of the most common causes of heart failure (HF), but with changing primary and secondary prophylaxis and increasing access to percutaneous interventions (PCI), additional factors are thought to be part of the HF pathophysiology after MI. One of the mechanisms is ischemia/reperfusion (I/R) injury.

I/R injury is a complex phenomenon in which, in the first stage of MI, there is a reduction or complete absence of blood flow in the coronary arteries, resulting in insufficient oxygen supply to the cardiomyocytes. After thrombolytic

✉ Ceren Eyileten
ceyileten@wum.edu.pl

1 Department of Experimental and Clinical Pharmacology, Medical University of Warsaw, Center for Preclinical Research and Technology (CePT), Warsaw, Poland

2 Department of Diabetology and Internal Medicine, Medical University of Warsaw, Warsaw, Poland

3 Doctoral School, Medical University of Warsaw, Warsaw, Poland

4 Genomics Core Facility, Centre of New Technologies, University of Warsaw, Warsaw, Poland

5 Department of Neurochemistry, Institute of Psychiatry and Neurology, Warsaw, Poland

6 Department of Internal Medicine, Division of Cardiology, Medical University of Graz, Graz, Austria

7 Division of Endocrinology and Diabetology, Cardiometabolic Trials Unit, Medical University of Graz, Graz, Austria

8 Department of Anesthesiology, Penn State Milton S. Hershey Medical Center, Penn State College of Medicine, PAHershey, United States of America

Published online: 18 March 2026

treatment and/or PCI, the vessel is unblocked, and arterial blood flow returns, mitochondrial oxidation turns back to its pre-MI state, but systolic function adapts more slowly, resulting in myocardial stunning [1].

Sirtuins (SIRTs) are a family of seven NAD+-dependent enzymes, participating in the post-translational regulation of protein synthesis. They are key regulators of several physiological and pathological processes, including ageing, cell proliferation, energy homeostasis, DNA repair, inflammation, oxidative stress or apoptosis [2]. SIRTs play an important role in the crosstalk between the environment and genome by modulating epigenetic mechanisms, mainly by histone modification [3]. Their expression varies widely among tissues. They are considered potential targets for the treatment of various diseases, including cardiovascular diseases (CVD) [4]. For many years, SIRTs were thought to play a protective role against aging, inflammation, and oxidative stress, thereby conferring cardioprotection in ischemia/reperfusion (I/R) injury [5, 6]. However, accumulating evidence indicates that these findings may be biased and suggests that not all SIRTs exert such protective effects; in fact, some members of the sirtuin family may lack cardioprotective activity or may even have context-dependent detrimental roles [7, 8].

Non-coding RNAs (ncRNAs), including microRNA (miRNA) and long non-coding RNA (lncRNA), play an essential role in gene expression regulation through transcription, RNA processing and translation [9]. Recent studies have already shown that ncRNAs can modulate CVDs progression by targeting SIRTs signalling [10, 11].

Several earlier reviews have examined sirtuin biology in CVDs, yet not all sirtuin isoforms have been comprehensively assessed within CV pathophysiology because of insufficient experimental data [12]. In parallel, the broader roles of miRNAs and lncRNAs in cardiac pathophysiology, aging, and metabolic regulation have also been extensively reviewed. However, these analyses generally treat sirtuin signaling or ncRNA regulation broadly, without concentrating on myocardial I/R injury as a unique pathological condition [13]. Furthermore, current publications largely lack a unified framework that links specific ncRNAs to particular sirtuin isoforms and distinct cellular mechanisms, such as apoptosis, oxidative stress, mitochondrial dysfunction, autophagy, and pyroptosis. Therefore, in this review paper, we aimed to investigate the role of ncRNAs regulating SIRTs pathways in I/R myocardial injury. For this purpose, we distinguish our work in several key aspects. First, we provide a focused review of preclinical evidence on ncRNA-SIRTs interactions particular focus on the context of myocardial I/R injury, rather than CVDs in general. Second, we systematically described experimentally studied miRNA-SIRT and lncRNA-SIRT regulatory axes that defined biological processes relevant to I/R injury. Third, we integrate these findings through bioinformatics analysis, using a literature-derived interaction network to identify highly connected ncRNAs, sirtuin isoforms, and downstream mediators that may represent key regulatory hubs.

## Article Search Process

Electronic databases (PubMed and Scopus) were searched during the period from June 2024 to January 2026, irrespective of article publication date, and original studies were reviewed to evaluate the role of ncRNAs involved in the SIRT pathways as potential biomarkers in I/R injury in myocardial infarction. Review articles were incorporated into this, as well as their secondary references for possible inclusion. Titles and abstracts were screened by two independent operators. The following search syntax was used: "Search ("miRNA" [MeSH Terms] OR "lncRNA" [MeSH Terms] AND "SIRT" [MeSH Terms] AND ("ischemia/reperfusion injury" [All Fields] OR "myocardial infarction" [All Fields]). Our search was limited to myocardial I/R injury only, we excluded the studies that evaluated other I/R diseases, such as ischemic stroke (Fig. 1).

## SIRTs Role in I/R Injury

SIRTs are a family of seven $NAD^+$-dependent deacetylases and ADP-ribosyltransferases that regulate a broad spectrum of cellular processes, including chromatin remodeling, transcriptional control, metabolism, mitochondrial function, oxidative stress responses, inflammation, and apoptosis [14]. In the CVD, SIRTs act as central stress sensors and metabolic regulators, integrating redox status and energy availability with gene-expression programs that determine cardiomyocyte survival during I/R injury [15].

Among them, SIRT1 is the most extensively studied isoform in the context of myocardial I/R. Cardiac-specific SIRT1 deficiency leads to larger infarct size and impaired functional recovery, whereas SIRT1 overexpression limits myocardial damage and improves post-ischemic cardiac performance [16]. Mechanistically, SIRT1 modulates multiple downstream pathways, including activation of antioxidant defenses via FoxO transcription factors and MnSOD, suppression of pro-apoptotic signaling through p53 deacetylation, and inhibition of inflammatory responses via NF-κB [17].

Importantly, recent studies have demonstrated that SIRT1 function in I/R is also regulated at the epigenetic and transcriptional level. Yang et al. (2017) showed that I/R and hypoxia/reoxygenation (H/R) induce trimethylation of histone H3K9 at the SIRT1 promoter through recruitment

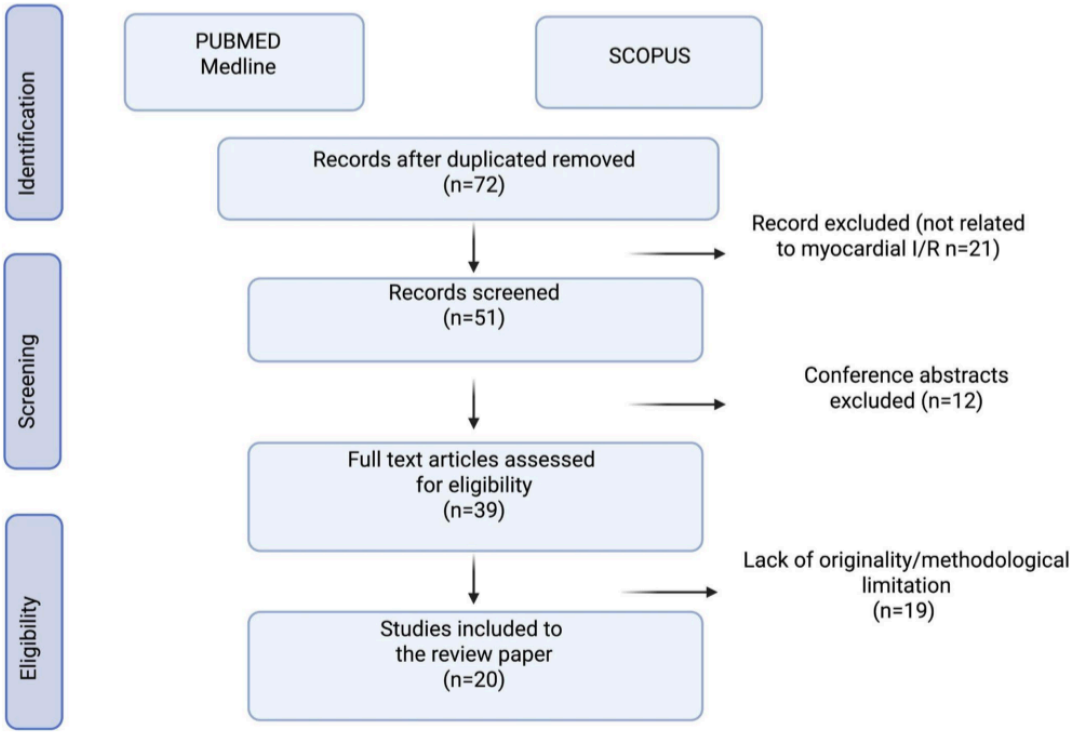


**Fig. 1** Article selection flowchart. The figure schematically depicts the article selection process. Created in https://BioRender.com

of the histone methyltransferase SUV39H1, leading to transcriptional repression of SIRT1. Genetic or pharmacological inhibition of SUV39H1 restored SIRT1 expression, reduced oxidative stress, limited infarct size and improved cardiac function in vivo, identifying the SUV39H1–SIRT1 axis as a novel epigenetic mechanism contributing to myocardial I/R injury [18].

In addition, interferon regulatory factor 9 (IRF9) has been identified as a key transcriptional modulator linking inflammatory signaling with SIRT1-dependent cardioprotection. Zhang et al. (2014) demonstrated that IRF9 is upregulated in human ischemic myocardium and in murine I/R models, and that genetic ablation of IRF9 attenuates cardiomyocyte apoptosis, inflammation and cardiac dysfunction. Mechanistically, IRF9 negatively regulates the SIRT1–p53 axis, and the protective effect of IRF9 deficiency is abolished in cardiomyocyte-specific SIRT1 knockout mice, indicating that IRF9 mediates myocardial I/R injury through suppression of SIRT1 signaling [19].

SIRT2 Although SIRT2 and SIRT4 are far less studied in myocardial I/R injury than other SIRT isoforms, recent clinical data suggest their potential relevance in prediction of post-infarction remodeling. A translational study in patients with acute myocardial infarction treated with the SGLT2 inhibitor empagliflozin showed that baseline levels of SIRT2 and SIRT4, together with selected sirtuin-related miRNAs, predicted impaired improvement of left ventricular function over 26 weeks; however, mechanistic experimental validation is still required to define their causal roles and downstream signaling in I/R injury [7]. Another clinical evidence further supports the potential relevance of SIRT2 in human myocardial injury. In a prospective cohort of patients with acute myocardial infarction, elevated plasma SIRT2 levels were independently associated with worse left ventricular function and a markedly increased risk of major adverse cardiovascular events and heart failure during follow-up, identifying SIRT2 as a promising prognostic biomarker in the post-infarction setting [20].

SIRT3, predominantly located in the mitochondria, is crucial for maintaining mitochondrial integrity and redox balance. Studies have shown that SIRT3 deficiency exacerbates I/R injury, characterized by increased oxidative damage and impaired energy metabolism. In contrast, SIRT3 overexpression preserves mitochondrial function, reduces infarct size,

**Table 1** Overview of the reviewed articles

| Ref | ncRNA | ncRNA targets | Outcome in the studied model | Methodology model | ncRNA - SIRT pathway |
|---|---|---|---|---|---|
| Lusha et al. [38] | ↓ miR-144 | FOXO1 | apoptosis | in vivo murine I/R model,, in vitro H/R model mimicking I/R injury in H9C2 cells | MiR-144 downregulates FOXO1, serving a protective role in I/R injury |
| Zhuo et al. [49] | ↑ miR-132 | SIRT1/PGC-1a/ Nrf2,NLRP3, caspase-1, IL-1B | pyroptosis, oxidative stress | in vivo mice I/R model, in vitro H/R model mimicking I/R injury in H9C2 cells | MiRNA-132 promotes oxidative stress-induced pyroptosis by targeting SIRT1 |
| Yao et al. [36]. | ↑ lncRNA MALAT1<br>↓ miR-217 | SIRT1 | apoptosis | in vitro H/R model mimicking I/R injury in H9C2 cells | MALAT1 protects against I/R injury via inhibition of miR-217 |
| Li et al. [51]. | ↓ miR-34a | ↓ p53<br>↑ SIRT1 | apoptosis | in vivo murine I/R model; in vitro H/R model mimicking I/R injury in H9C2 cells | Metformin exerts its anti-apoptotic effects in cardiac cells through the modulation of the p53/miR-34a/ SIRT1 signalling pathway |
| Zhang et al. [50]. | ↑ miR-9-5p | ↓ SOCS5<br>↓ SIRT1<br>↑ JAK2/STAT3<br>↑ NF-κB | myocardial infarction size, inflammation | in vivo murine I/R model,<br>in vitro validation in human serum | miR-9-5p promotes neutrophil polarization toward the pro-inflammatory N1 phenotype |
| Xu et al. [47]. | ↑ miR-15b-5p | ↓ SIRT3<br>↑ NLRP3<br>↑ IL-1β<br>↑ IL-18<br>↑ LDH<br>↑ caspase-1 | myocardial infarction size, pyroptosis | in vivo rat I/R model, in vitro H/R model mimicking I/R injury in H9C2 cells | Inhibition of miR-15b-5p or overexpression of SIRT3 mitigated pyroptosis and attenuated the ischemic heart injury |
| Wang et al. [35]. | ↓ lncRNA Peg13 | ↓ miR-34a<br>↑ SIRT1<br>↓ Bax<br>↓ caspase 3<br>↓ GRP78<br>↓ CHOP<br>↑ Bcl-2<br>↑ Peg13 | myocardial infarction size, apoptosis | in vivo murine I/R model, in vitro H/R model mimicking I/R injury in HL-1 and 293T cells | Overexpression of Peg13 attenuated endoplasmic reticulum stress and apoptosis, reducing the infarct size and preserving cardiac function |
| Sun et al. [27] | ↑ miR-148b-3p | ↓ SIRT7/p53<br>↑ LDH<br>↑ Puma<br>↑ Bax | apoptosis | in vitro H/R model mimicking I/R injury in cardiomyocytes (C57BL6 mice) | miR-148-3p was upregulated in response to I/R enhancing apoptosis, SIRT7 overexpression rescued miR-148-3p induced cell apoptosis |
| Song et al. [34]. | ↑ lncRNA ANRIL<br>↓ miR-181a | ↑ SIRT1<br>↓ Bax<br>↑ Bcl-2<br>↓ caspase-3<br>↓ LDH | apoptosis | in vitro H/R model mimicking I/R injury in H9C2 cells | ANRIL upregulates SIRT1 expression by sponging miR-181a, protecting H9c2 cells from apoptosis and oxidative stress |
| Shu et al. [33]. | ↑ lncRNA ANRIL<br>↓ miR-7-5p | ↑ SIRT1<br>↓ Bax<br>↑ Bcl-2<br>↓ caspase-3<br>↓ caspase-9 | apoptosis, oxidative stress | in vitro H/R model mimicking I/R injury in H9C2 cells | ANRIL upregulates SIRT1 expression by sponging miR-7-5p, protecting H9c2 cells from apoptosis and oxidative stress |
| Qiu et al. [41]. | ↓ miR-204 | ↓ SIRT1<br>↓ Bax<br>↑ Bcl-2<br>↓ LC3-II/LC3-I<br>↓ Beclin-1 | apoptosis, autophagy | in vitro H/R model mimicking I/R injury in H9C2 cells | miR-204 overexpression downregulated SIRT1 and inhibited apoptosis and autophagy |
| Qi et al. [40]. | ↑ miR-217-5p | ↓ SIRT1<br>↓ LC3<br>↓ PINK1<br>↓ Parkin<br>↓ Bcl-2<br>↓ c-IAP<br>↑ p62<br>↑ caspase-3 | apoptosis, mitochondrial function | in vitro H/R model mimicking I/R injury in H9C2 cells | Silencing miR-217 resulted in the restoration of mitochondrial function via upregulation of SIRT1 expression |
| Qi et al. [32]. | ↑ miR-181a-5p | ↓ SIRT1<br>↓ Bcl-2<br>↑ Bax<br>↑ Caspase 3 | apoptosis, oxidative stress | in vitro H/R model mimicking I/R injury in H9C2 cells | Downregulated miR-181a-5p promoted cell viability; SIRT1 counteracted negative effects of overexpressed miR-181a-5p |

**Table 1** (continued)

| Ref | ncRNA | ncRNA targets | Outcome in the studied model | Methodology model | ncRNA - SIRT pathway |
|---|---|---|---|---|---|
| Niu et al. [42] | ↓ Oip5-as1<br>↑ miR-29a | ↑ SIRT1<br>↑ AMPK/PGC1a | apoptosis, oxidative stress | in vivo murine I/R model, in vitro H/R model mimicking I/R injury in H9C2 cells | Oip5-as1 suppresses miR-29a leading to activation of protective SIRT1/AMPK/PGC1a pathway |
| Ning et al. [39] | ↓ miR-494 | ↑ Pi3K/AKT/mTOR<br>↑ SIRT1 | apoptosis, autophagy | in vitro H/R model mimicking I/R injury in H9C2 cells | miR-494 targeted SIRT1, which alleviated cell apoptosis and autophagy |
| Fu et al. [31]. | ↑ miR 34a | ↓ SIRT1<br>↓ Bcl-2<br>↑ Bax<br>↑ p53 | left ventricular ejection fraction | in vivo rat I/R model, in vitro H/R model mimicking I/R injury in NRCMs | miR-34a overexpression exacerbated I/R injury; SIRT1 was negatively regulated by miR-34a |
| Du et al. [46] | ↑ miR-22 | ↓ SIRT1<br>↓ PGC1a | myocardial infarct size, apoptosis, oxidative stress, mitochondrial function | in vivo rat I/R model, in vitro H/R model mimicking I/R injury in H9C2 cells | miR-22 inhibition demonstrate therapeutic potential by silencing of Sirt1 |
| Ding et al. [48] | ↑ miR-29a | ↓ SIRT1<br>↑ NLRP3<br>↑ caspase-1<br>↑ IL-1b<br>↑ iNOS<br>↑ MDA | oxidative stress, pyroptosis | in vivo C57BL/J6 mice I/R model; in vitro H/R model mimicking I/R injury in H9C2 cells | inhibition of miR-29a improved myocardial I/R injury by upregulating the SIRT1 expression |
| Chen et al. [29] | ↓ miR-30c-5p | ↓ SIRT1<br>↑ NF-κB<br>↑ Bax<br>↑ caspase-3 | apoptosis | in vivo rat I/R model; in vitro rat cardiac myocytes, | miR-30c-5p downregulates SIRT1, resulting in enhanced apoptosis |
| Chen et al. [28]. | ↓ miR-125b | ↓ Bax<br>↓ caspase-3<br>↓ SIRT7<br>↓ IL-1β, IL-6, TNF-α<br>↑ Bcl-2 | apoptosis, inflammation | in vivo rat I/R model;<br>in vitro rat cardiac myocytes | miR-125b downregulates SIRT7, which inhibits apoptosis and inflammation, alleviating the I/R injury |

*Abbreviations: AKT* Protein kinase B, *AMPK* AMP-activated protein kinase, *Bax* BCL2 associated X, apoptosis regulator, *Bcl-2* B-cell lymphoma 2, *c-IAP* Cellular inhibitor of apoptosis protein, *caspase-1* Cysteine-aspartic acid protease 1, *caspase-3* Cysteine-aspartic acid protease 3, *caspase-9* Cysteine-aspartic acid protease 9, *CHOP* C/EBP homologous protein, *FOXO1* Forkhead box protein O1, *GRP78* Glucose-regulated protein 78, *IL-18* Interleukin 18, *IL-1B* Interleukin 1 beta, *IL-6* Interleukin 6, *iNOS* Inducible nitric oxide synthase, *JAK2* Janus kinase 2, *LC3* Microtubule-associated protein 1A/1B-light chain 3, *LDH* Lactate dehydrogenase, *MDA* Malondialdehyde, *NF-κB* Nuclear factor kappa-light-chain-enhancer of activated B cells, *NLRP3* NOD-, LRR- and pyrin domain-containing protein 3, *Nrf2* Nuclear factor erythroid 2-related factor 2, *mTOR* Mammalian target of rapamycin, *miR* MicroRNA, *PGC-1a* Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *PINK1* PTEN-induced kinase 1, *Parkin* E3 ubiquitin-protein ligase PARK2, *Peg13* Paternally expressed gene 13, *Pi3K* Phosphoinositide 3-kinase, *Puma* p53 upregulated modulator of apoptosis, *SIRT1* Sirtuin 1, *SIRT3* Sirtuin 3, *SIRT7* Sirtuin 7, *SOCS5* Suppressor of cytokine signaling 5, *STAT3* Signal transducer and activator of transcription 3, *TNF-α* Tumor necrosis factor alpha

and limits cardiomyocyte apoptosis. These protective effects are primarily mediated by SIRT3's ability to deacetylate and activate key mitochondrial enzymes involved in the detoxification of reactive oxygen species (ROS) and energy production [21]. Thus, SIRT3 is considered as a key component of the mitochondrial stress response in ischemic heart disease.

More recently, SIRT5 has been implicated in the regulation of mitochondrial quality control and redox balance during I/R injury. Li et al. (2025) showed that the transcription factor HOXB5 activates SIRT5, thereby improving mitochondrial function, reducing ROS production and promoting mitophagy, whereas SIRT5 knockdown abolishes these cardioprotective effects [22].

SIRT6 has also been shown to exert potent cardioprotective effects in I/R injury. Wang et al. (2016) demonstrated that SIRT6 deficiency exacerbates oxidative stress and cardiac dysfunction, whereas SIRT6 overexpression activates the AMPK–FoxO3α pathway, leading to transcriptional upregulation of antioxidant enzymes such as MnSOD and catalase and enhanced reactive oxygen species scavenging [6].

Recent studies further support the importance of mitochondrial, redox and inflammatory pathways overlapping with sirtuin signaling, showing that kirenol-mediated NOX1/NOX4 inhibition [23] and MIF-driven activation of AMPK and ERK1/2 pathways [24] significantly reduce infarct size, pyroptosis and apoptosis, and improve cardiac function in experimental I/R models.

Although the role of SIRT7 in I/R injury is not yet fully elucidated, recent evidence points to its involvement in regulating apoptosis and cellular stress responses. Downregulation of SIRT7, mediated by miRNAs such as miR-148b-3p, has been linked to enhanced cardiomyocyte

apoptosis, whereas restoring its expression improves cell viability. Despite limited studies, SIRT7 is considered a potential therapeutic target, warranting further investigation in the context of I/R injury [25].

Collectively, these studies demonstrate that sirtuins coordinate a complex network of epigenetic, metabolic, mitochondrial, and inflammatory pathways that critically govern myocardial susceptibility to ischemia/reperfusion injury. In the subsequent sections, we delineate how miRNAs and lncRNAs modulate these sirtuin-dependent signaling cascades, thereby fine-tuning cardiomyocyte survival, inflammatory responses, and post-ischemic remodeling **(**Table 1**).**

## Regulation of Apoptosis and Cardiomyocyte Survival

Cardiomyocyte apoptosis represents a central mechanism of myocardial damage during ischemia/reperfusion, contributing directly to infarct expansion and subsequent ventricular dysfunction. This process is tightly controlled by sirtuin-dependent signaling pathways, particularly those involving SIRT1 and SIRT7, which modulate key regulators of cell death such as Bax, Bcl-2, caspases, p53 and FOXO1. Increasing evidence indicates that both microRNAs and long non-coding RNAs fine-tune these apoptotic pathways by targeting sirtuins or acting as competing endogenous RNAs, thereby critically influencing cardiomyocyte survival under ischemic stress [26].

The miR-148b-3p promotes apoptosis in H/R injured cardiomyocytes by targeting SIRT7 and modulation of the proapoptotic p53 pathway. Inhibition of miR-148b-3p improved cell viability, reduced apoptosis, and enhanced SIRT7 expression. These findings indicate that miR-148b-3p modulates SIRT7/p53 signaling and suggest its inhibition as a strategy to protect against myocardial I/R injury [27].

One study investigated that miR-125 is downregulated in cardiomyocytes retrieved from the I/R injury rat model, which is followed by increased apoptosis. The proapoptotic proteins, namely Bax and caspase-3 were overexpressed in I/R heart tissue. Also, the SIRT7 expression, which is the regulator of apoptosis and stress response, was significantly higher. The cardiomyocyte culture treatment with exosomes delivered from bone marrow mononuclear cells (BMCs) previously transfected with miR-125b alleviated the apoptosis and downregulated the expression of Bax, caspase-3, and SIRT7. The BMSC-Exo-125b may inhibit the inflammation and apoptosis in I/R myocardium cells by targeting SIRT7, which puts it as a future therapeutic target [28].

Building on this, later investigations analyzed the pro-inflammatory properties of miR-30c in I/R injury. Following I/R injury in rats, miR-30c expression was markedly upregulated in their cardiac tissues, which coincided with increased apoptosis and the activation of Bax, caspase-3, and NF-κB p65 signaling. Introduction of miR-30c-5p mimics markedly suppressed SIRT1 expression in cardiomyocytes at both the transcript and protein levels, whereas inhibition of miR-30c-5p produced the opposite effect. Notably, the pro-survival actions of the miR-30c-5p inhibitor on apoptosis and NF-κB signaling were abolished upon SIRT1 silencing, indicating that SIRT1 is a critical mediator of the protective effects conferred by miR-30c-5p inhibition. In addition, knockdown of SIRT1 itself further elevated the apoptotic index and enhanced the expression of Bax, caspase-3, and phosphorylated NF-κB p65. These results suggest that miR-30c-5p promotes apoptosis and inflammation by inhibiting SIRT1 in I/R injury in cardiomyocytes [29].

Moreover, it was shown that higher expression of miR-34a in the endothelial progenitor cell obtained from patients with coronary artery disease (CAD) are associated with the endothelial damage by down-regulating the expression of *SIRT1* gene [30]. Later, it was demonstrated that inhibition of miR-34a reduces in vitro apoptosis in cardiomyocytes following hypoxia/reoxygenation (H/R) injury of cardiomyocytes mimicking I/R injury. This effect was mediated through the upregulation of SIRT1 and Bcl-2 and the downregulation of ac-p53 and Bax. Consequently, this modulation resulted in an increased Bcl-2/Bax ratio, a well-established marker of apoptosis, indicative of reduced apoptotic activity. In vivo analysis supported the protective effect of miR-34a inhibition, as blocking miR-34a ameliorated the recovery of left ventricular function and decreased I/R injury [31].

Another study demonstrated that miR-181a-5p is upregulated in H/R injury in cardiomyocytes. MiR-181a-5p targets SIRT1, resulting in increased apoptosis and oxidative stress. Downregulation of miR-181a-5p improved cell viability, ROS, and decreased apoptosis markers such as Bax and caspase-3. The study concluded that miR-181a-5p is a key mediator of H/R induced cardiomyocyte injury via SIRT1 regulation, making it a potential therapeutic target [32].

The role of lncRNA ANRIL in H/R injury in cardiomyocytes was studied. ANRIL upregulated SIRT1 expression by sponging miR-7-5p, via protecting H9c2 cells from apoptosis and oxidative stress. Silencing ANRIL exacerbated H/R injury, while overexpression ameliorated it, highlighting the protective potential of the ANRIL/miR-7-5p/SIRT1 axis [33]. Another research study extended these findings by exploring the protective action of ANRIL in cardiomyocytes against H/R injury by sponging miR-181a, thereby enhancing SIRT1 expression. ANRIL overexpression reduced apoptosis and lactate dehydrogenase (LDH) release, while miR-181a overexpression or SIRT1 knockdown negated these effects. The ANRIL/miR-181a/SIRT1 axis was identified as a novel mechanism for mitigating myocyte H/R injury [34].

Additionally, the lncRNA Peg13 in myocardial I/R mice model injury was studied. Peg13 expression was significantly reduced in I/R treated cardiac tissues and cardiomyocytes. Overexpression of Peg13 attenuated endoplasmic reticulum (ER) stress and apoptosis, reducing the infarct size and preserving cardiac function in vivo. Mechanistically, Peg13 upregulated SIRT1 expression by sponging miR-34a, thereby alleviating ER stress and apoptosis. These findings can show Peg13 as a promising therapeutic target for mitigating myocardial I/R injury through modulation of the miR-34a/SIRT1 pathway [35].

Further studies on H/R injury showed that hypoxia upregulated the expression of the lncRNA MALAT1, which in turn negatively regulated miR-217 via direct binding. Knockdown of MALAT1 exacerbated H/R injury through enhanced apoptosis and further loss of cell function, effects mediated by miR-217 overexpression. Conversely, silencing of miR-217 rescued cell viability and attenuated apoptosis, which confirms a functional MALAT1/miR-217 interaction. Further analyses identified *SIRT1* as a direct downstream target of miR-217. Hypoxia elevated *SIRT1* expression, while miR-217 overexpression suppressed hypoxia. The overexpression of *SIRT1* significantly alleviated hypoxia-induced cell injury, while its knockdown exacerbated cellular damage. SIRT1-mediated protection was linked to reactivation of PI3K/AKT and Notch signaling pathways. Hypoxia inhibited phosphorylation of PI3K and AKT as well as Notch receptor expression, whereas *SIRT1* overexpression restored their activity. Collectively, these findings identify the MALAT1/miR-217/SIRT1 axis and its downstream signaling via PI3K/AKT and Notch as critical regulators of H/R cardiomyocyte injury [36].

FOXO1 is a transcription factor involved in the regulation of oxidative stress, apoptosis, and metabolism. In cardiomyocytes, FOXO1 activity is tightly controlled under physiological conditions, but its dysregulation during I/R contributes to cellular injury and impaired cardiac function [37]. Another miRNA, namely miR-144 was significantly downregulated in myocardial I/R in vivo and H/R in vitro models. Functional experiments showed that overexpression of miR-144 markedly reduced infarct size, cardiomyocyte apoptosis, and levels of myocardial injury markers (CK, LDH) in I/R rats, without exerting effects in the absence of ischemic injury. In vitro, miR-144 overexpression in H9c2 cells significantly decreased H/R induced apoptosis and oxidative stress, while its silencing had no effect under normoxic conditions. Mechanistically, FOXO1 was identified as a direct target of miR-144 using luciferase reporter assays and confirmed at both mRNA and protein levels. Overexpression of FOXO1 reversed the cytoprotective effects of miR-144, reinstating apoptosis and elevating CK and LDH levels. Finally, FOXO1 expression was found to be upregulated in injured myocardium and cells, and was inversely correlated with miR-144 expression in myocardial tissues. These findings establish the miR-144/FOXO1 axis as a key regulator of cardiomyocyte survival under ischemic conditions, providing a potential therapeutic target for myocardial I/R injury [38].

Collectively, these studies demonstrate that both miRNAs and lncRNAs critically regulate cardiomyocyte apoptosis and survival during I/R injury through modulation of SIRT1 and SIRT7-dependent pathways, converging on key apoptotic regulators such as Bax, Bcl-2, caspase-3, p53 and FOXO1. Targeting these pathways represents a promising therapeutic strategy to alleviate apoptosis and improve myocyte survival after myocardial ischemic injury **(**Table 1**).**

## Control of Oxidative Stress, Mitochondrial Function and Autophagy

Beyond apoptosis, myocardial I/R injury involves profound disturbances in mitochondrial homeostasis, redox balance and autophagic flux. Sirtuins, particularly SIRT1 and emerging isoforms such as SIRT5, play pivotal roles in regulating mitochondrial metabolism, antioxidant defense and quality control pathways, including AMPK/PGC-1α, PI3K/AKT/mTOR and mitophagy signaling. Recent studies reveal that these processes are tightly modulated by specific ncRNAs, which act upstream of sirtuins to orchestrate cellular adaptation to ischemic stress.

The role of miR-494 in cardiomyocyte apoptosis and autophagy induced by H/R was studied. The miR-494 is significantly downregulated during I/R, leading to increased apoptosis and autophagy via targeting SIRT1. Overexpression of miR-494 suppressed H/R induced apoptosis and autophagy by stimulating the PI3K/AKT/mTOR axis, while inhibition of miR-494 exacerbated these effects. Knockdown of SIRT1 partially reversed the protective role of miR-494. This study highlights miR-494 as a critical regulator and potential therapeutic target for myocardial H/R injury [39].

Additionally, the role of miR-217-5p in mitochondrial dysfunction and apoptosis in H/R injury was studied. As miR-217-5p targets SIRT1, impairs mitochondrial function, and increases apoptosis via activating the p62 and cleaved caspase-3 pathways. Inhibition of miR-217-5p restored mitochondrial function and protected against H/R-induced cardiomyocyte injury. These findings emphasise the therapeutic potential of targeting the miR-217-5p/SIRT1 axis [40].

Besides, a previous study reported that miR-204 expression is downregulated in cardiomyocytes subjected to H/R injury. Treatment of cardiomyocytes with miR-204 led to downregulation of SIRT1 expression and inhibition of both

apoptosis and autophagy through modulation of the Bax/Bcl-2 signaling pathway. Notably, SIRT1 overexpression ameliorated the suppressive effects of miR-204 overexpression on autophagy and apoptosis, highlighting the potential role of the miR-204/SIRT1 axis in mediating cardioprotection during myocardial I/R injury [41].

Furthermore, lncRNA Oip5-as1 in myocardial I/R injury was investigated. Oip5-as1 expression was found to be significantly reduced in myocardial tissues obtained from rats after I/R as well as in cells exposed to H/R conditions. The Oip5-as1 alleviated oxidative stress and apoptosis by sponging miR-29a, activating the SIRT1/AMPK/PGC1α pathway. Downregulation of Oip5-as1 led to increased oxidative stress and apoptosis, while its overexpression conferred protection against I/R injury. These results suggest a pivotal role of the Oip5-as1/miR-29a/SIRT1 axis in cardioprotection after I/R injury [42].

MiR-22 is a highly expressed miRNAs both in cardiac and skeletal muscles, which is significantly up-regulated during cardiac aging and fibrosis [43] and inhibition of miR-22 conducted cardioprotection against oxidative stress [44]. Previous studies predicted that inhibition of miR-22 can have a protective role against mitochondrial oxidative injury by directly targeting SIRT1 and peroxisome proliferator-activated receptor-coactivator-1α (PGC1α) [45, 46]. Subsequent studies confirmed the role of miR-22 in an animal I/R model. The expression of miR-22 in myocardial I/R injury was upregulated, which can be due to ROS-induced activation of p53. On the other hand, significantly decreased infarct size in I/R rats treated with miR-22 inhibitor was observed. Moreover, in vitro analysis confirmed the previous prediction and showed that miR-22 leads to mitochondrial dysfunction and cell injury via targeting the SIRT1/PGC1α axis. Therefore, the study highlighted the miR-22 inhibitor as a potential therapeutic target for acute myocardial I/R injury by maintaining cardiac mitochondrial function [46].

Taken together, the available evidence highlights ncRNA-dependent regulation of sirtuins as a critical determinant of mitochondrial function, oxidative stress responses and autophagy during I/R injury. By modulating key metabolic and redox signaling pathways, these ncRNA–SIRT networks substantially influence cardiomyocyte survival and functional recovery what underscores their therapeutic potential **(**Table 1**).**

## Regulation of Pyroptosis and Inflammatory Signaling

Pyroptosis and excessive inflammatory signaling are increasingly recognized as key contributors to myocardial ischemia/reperfusion injury, amplifying cardiomyocyte loss and adverse remodeling. These processes are tightly linked to activation of the NLRP3 inflammasome, oxidative stress pathways, and cytokine release, and are critically modulated by sirtuin-dependent signaling. Recent studies demonstrate that specific microRNAs and extracellular vesicle–derived ncRNAs regulate these inflammatory and pyroptotic responses through targeting SIRT1 and SIRT3, thereby shaping both cardiomyocyte fate and immune cell behavior during I/R injury.

Xu et al. demonstrated that H/R injury induces pyroptosis in H9C2 cardiomyocytes by upregulating miR-15b-5p and activating the NLRP3 inflammasome pathway. H/R exposure led to significantly elevated miR-15b-5p levels, decreased cell viability, increased LDH release, and higher concentrations of proinflammatory cytokines IL-1β and IL-18. Concomitantly, NLRP3 expression and pyroptosis markers, cleaved Caspase-1 and apoptosis-associated speck-like protein (ASC) were markedly upregulated, indicating inflammasome activation. Knockdown of miR-15b-5p reversed these effects, improving cell viability and attenuating inflammasome activation and pyroptosis, thereby implicating miR-15b-5p as a pro-pyroptotic mediator. Additionally, in silico and in vitro analysis showed miR-15b-5p as SIRT3 inhibitor. H/R exposure suppressed SIRT3 expression, which was rescued upon miR-15b-5p inhibition. SIRT3 overexpression mimicked the protective effects of miR-15b-5p silencing, while its knockdown abolished them, confirming its critical role in mediating pyroptosis via the NLRP3 pathway. In vivo, miR-15b-5p was upregulated in a rat model of myocardial I/R injury, accompanied by downregulation of SIRT3, increased myocardial infarct size, elevated serum cTnT, and enhanced inflammasome-related protein expression. Intramyocardial injection of miR-15b-5p antagomir mitigated myocardial injury, reduced infarct area and inflammatory cytokines, and restored SIRT3 levels. These findings collectively suggest that the miR-15b-5p/SIRT3/NLRP3 axis plays a pivotal role in I/R pathophysiology and may represent a potential therapeutic target [47].

Another study explored that miR-29a expression is upregulated in the mice cardiomyocytes after I/R injury and the mRNA and protein levels of SIRT1 were significantly down-regulated. The pyroptosis-related proteins (NLRP3, caspase-1, and IL-1b), and oxidative stress-related proteins (iNOS, MDA) were overexpressed after I/R injury. I/R injury activated oxidative stress and pyroptosis via increased expression of miR-29a and decreased expression of SIRT1. Additionally, in vitro inhibition of miR-29a alleviated oxidative stress and pyroptosis and upregulated the expression of SIRT1. The findings indicate that inhibiting miR-29a provides a protective effect by stimulating the SIRT1 pathway, highlighting it as a promising target for therapeutic intervention. This effect is mediated through restoration of SIRT1-dependent suppression of oxidative stress and pyroptotic signaling cascades [48].

I/R injury can be characterized by elevated serum cardiac enzymes (CK, CK-MB, LDH, HBDH, IMA) and inflammatory cytokines (IL-1β, IL-6, TNF-α), alongside histological abnormalities. Mechanistically, I/R increased miR-132 expression and downregulated SIRT1, leading to suppression of the PGC-1α/Nrf2 antioxidant signaling pathway and enhanced oxidative stress, as shown by altered levels of MDA and SOD. Pyroptosis was also activated, with upregulation of NLRP3, caspase-1, and IL-1β. In vitro H/R model confirmed SIRT1 as a direct target of miR-132. Inhibition of miR-132 restored SIRT1 expression, reactivated PGC-1α/Nrf2 signaling, reduced oxidative stress and pyroptosis. These results establish the miR-132/SIRT1/PGC-1α/Nrf2 axis as a key regulator of I/R induced myocardial injury [49].

Zhang et al. examined the role of cardiomyocyte-derived extracellular vesicle miR-9-5p in regulating neutrophil polarization during myocardial I/R injury. The findings demonstrated that miR-9-5p drives neutrophils toward a pro-inflammatory N1 phenotype through suppression of SOCS5 and SIRT1, leading to activation of the JAK2/STAT3 and NF-κB signaling pathways. This shift in polarization aggravated myocardial damage, resulting in larger infarct size and impaired cardiac function. Moreover, elevated circulating levels of EV-associated miR-9-5p were found to be linked with higher cardiovascular mortality in patients with ST-segment elevation myocardial infarction. These findings highlight the miR-9-5p/SOCS5/SIRT1 axis as a potential therapeutic and prognostic target in myocardial I/R injury [50].

In summary, miRNA-mediated modulation of SIRT1 and SIRT3-dependent pathways critically governs NLRP3 inflammasome activation and pyroptotic cell death in myocardial I/R injury. Restoration of sirtuin activity through inhibition of pro-pyroptotic miRNAs attenuates inflammatory cytokine release and limits tissue damage, underscoring these axes as promising therapeutic targets (Table 1).

## Future Perspective and Conclusion

This review highlights sirtuins as central regulators of myocardial ischemia/reperfusion injury, integrating metabolic, redox, apoptotic, and inflammatory signaling. In particular, SIRT1, SIRT3, and SIRT7 emerge as key nodal points through which microRNAs and long non-coding RNAs modulate cardiomyocyte survival, mitochondrial function, and immune responses (Fig. 2). Specific miRNAs, including miR-125b, miR-30c, miR-34a, miR-181a, and miR-29a, were shown to fine-tune sirtuin expression and downstream pathways, thereby influencing apoptosis, oxidative stress, pyroptosis, and post-ischemic remodeling. Conversely, lncRNAs such as ANRIL, MALAT1, and Peg13 exert cardioprotective effects by acting as competing endogenous RNAs and restoring SIRT1-dependent signaling, ultimately enhancing cellular resilience to ischemic stress (Fig. 3). To visualize the literature findings, we also performed interaction network based on literature search from Table 1. Table 1 was transformed in R and visualised in Cytoscape.

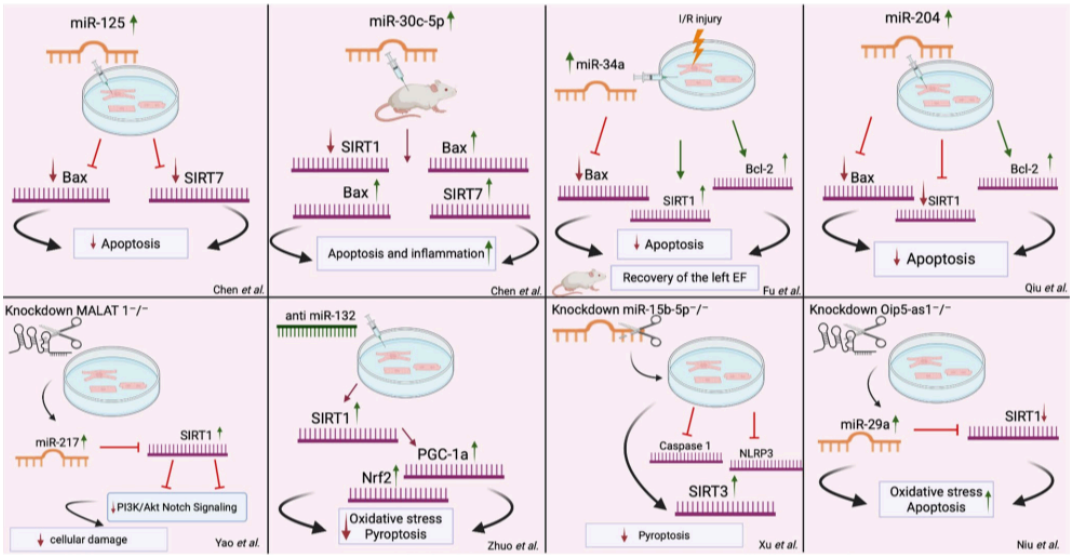


**Fig. 2** NcRNAs as therapeutic targets: Suppression or augmentation. Created in https://BioRender.com

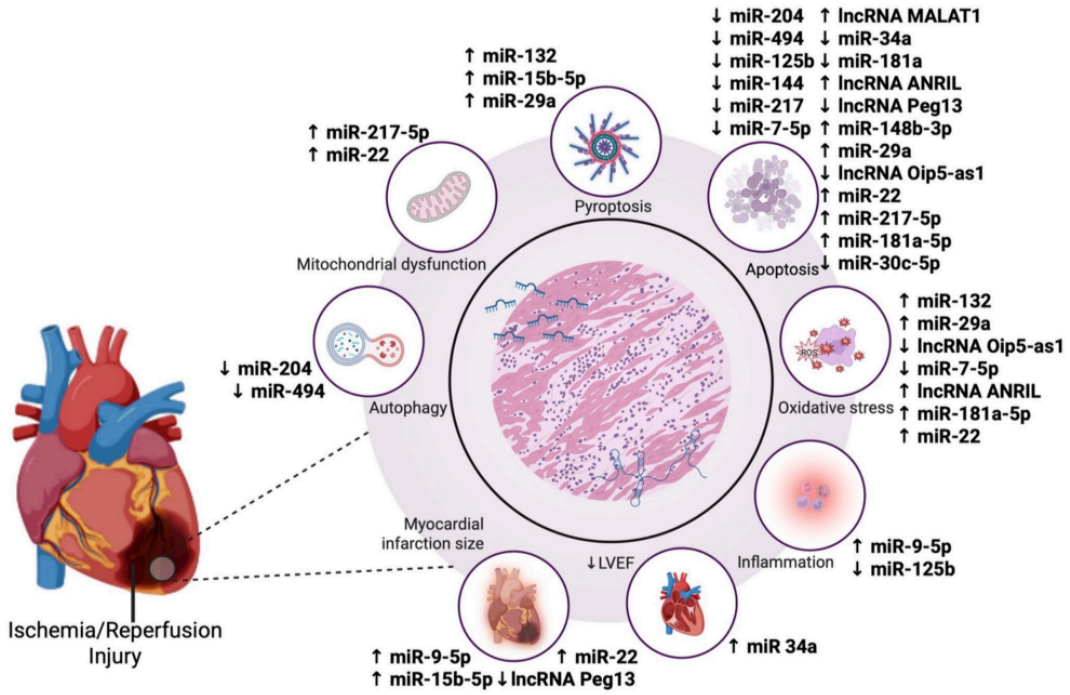


**Fig. 3** MiRNAs and lncRNAs alterations in I/R injury based on published data. ↑ Indicates the up-regulation during I/R injury, ↓ indicates the down-regulation during I/R injury. Created in https://BioRender.com

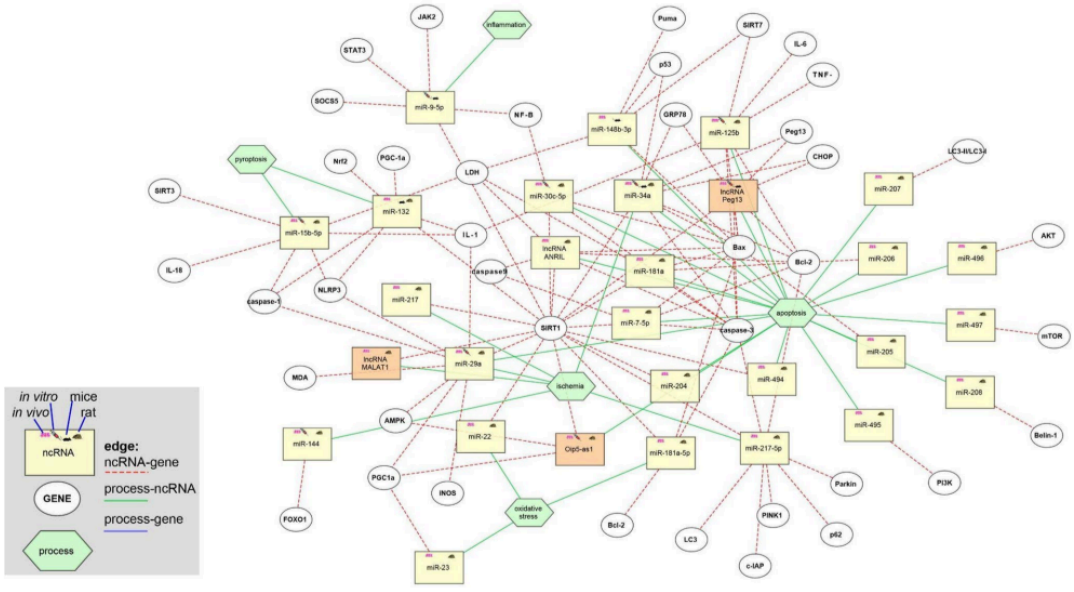


**Fig. 4** The interaction network between ncRNAs and specific genes regarding processes such as ischemia, pyroptosis, inflammation, oxidative stress, and apoptosis. Figure was generated based on Table 1

The edges and nodes were generated based on ncRNA-target-process interactions from the table. It allowed us better evaluate important interactions. We identified apoptosis as a process affecting the highest number of genes and miRNAs (19 interactions). Top miRNAs with the highest connectivity were miR-29a, miR-34a, and miR-217-5p. Top lncRNA was lncRNA Peg13. We also identified top regulatory genes SIRT1, Bax, caspase-3, and Bcl-2 based on the number of their connections with ncRNAs and processes (Fig. 4).

Although the currently available literature is still largely derived from preclinical models, the existing body of evidence provides a compelling rationale for continued investigation of sirtuin-centered regulatory networks in myocardial I/R injury. The recurrent involvement of key axes converging on SIRT1, SIRT3, and SIRT7 underscores the biological relevance of these pathways and highlights their promise as therapeutic and biomarker targets. Importantly, these findings pave the way for future translational and clinical studies aimed at validating ncRNA–sirtuin interactions in human I/R injury and at determining their utility in predicting outcomes or modulating cardioprotection.

At the same time, myocardial I/R injury is a multicellular process, and future work should extend beyond cardiomyocytes to systematically address ncRNA-sirtuin signaling in endothelial cells, fibroblasts, and immune cells, as well as their intercellular communication. These compartments play pivotal roles in inflammation resolution, fibrotic remodeling, and long-term ventricular function after myocardial infarction, yet remain largely unexplored in the context of ncRNA–sirtuin regulation.

Finally, expanding research to additional members of the sirtuin family and integrating mechanistic studies with well-phenotyped clinical cohorts will be essential to fully exploit the therapeutic and prognostic potential of sirtuin–miRNA/lncRNA networks in myocardial I/R injury.

## Abbreviations

| | |
|---|---|
| AKT | Protein kinase B |
| AMPK | AMP–activated protein kinase |
| Bax | BCL2 associated X, apoptosis regulator |
| Bcl | 2–B–cell lymphoma 2 |
| CAD | Coronary artery disease |
| caspase | 1–Cysteine–aspartic acid protease 1 |
| caspase | 3–Cysteine–aspartic acid protease 3 |
| caspase | 9–Cysteine–aspartic acid protease 9 |
| IAP | Cellular inhibitor of apoptosis protein |
| CHOP | C/EBP homologous protein |
| CK | Creatine Kinase |
| CK | MB–Creatine Kinase–Myocardial Band |
| FOXO1 | Forkhead box protein O1 |
| GRP78 | Glucose–regulated protein 78 |
| HBDH | Hydroxybutyrate Dehydrogenase |
| IL | 18–Interleukin 18 |
| IL | 1B–Interleukin 1 beta |
| IL | 6–Interleukin 6 |
| IMA | Ischemia–Modified Albumin |
| iNOS | Inducible nitric oxide synthase |
| I/R | ischemia reperfusion |
| JAK2 | Janus kinase 2 |
| LC3 | Microtubule–associated protein 1 A/1B–light chain 3 |
| LDH | Lactate dehydrogenase |
| MDA | Malondialdehyde |
| MI | Myocardial Infarction |
| miR | MicroRNA |
| mTOR | Mammalian target of rapamycin |
| NF | κB–Nuclear factor kappa–light–chain–enhancer of activated B cells |
| NLRP3 | NOD–, LRR–and pyrin domain–containing protein 3 |
| Nrf2 | Nuclear factor erythroid 2–related factor 2 |
| PGC | 1a–Peroxisome proliferator–activated receptor gamma coactivator 1–alpha |
| PINK1 | PTEN–induced kinase 1 |
| Parkin | E3 ubiquitin–protein ligase PARK2 |
| Peg13 | Paternally expressed gene 13 |
| Pi3K | Phosphoinositide 3–kinase |
| Puma | p53 upregulated modulator of apoptosis |
| SIRT1 | Sirtuin 1 |
| SIRT3 | Sirtuin 3 |
| SIRT7 | Sirtuin 7 |
| SOCS5 | Suppressor of cytokine signaling 5 |
| STAT3 | Signal transducer and activator of transcription 3 |
| TNF | α–Tumor necrosis factor alpha |

**Acknowledgements** This work was written by the members (ANS, CE, ZW, DvL, HS, MP) of the International and Intercontinental Cardiovascular and Cardiometabolic Research Team (I-COMET; www.icomet.science).

**Author Contributions** Writing - original draft preparation, ANS, CE, ZW, SA, MP; writing - review and editing, ANS, CE, ZW, DvL, HS, MP; bioinformatic analysis, ZW; visualization, ANS, CE, ZW; supervision, CE, MP. All authors have read and agreed to the published version of the manuscript.

**Funding** ANS was supported financially as part of the research grant from Polish National Science Center (NCN), Poland (grant number 2022/45/N/NZ7/0246) and NAWA STER programme (BPI/STE/2024/1/00027/U/00001). CE was supported by the NAWA/Walczak programme (BPN/WAL/2024/1/00057). and MP by the Polish Medical Research Agency (ABM) (grant number 2019/ABM/01/00037). HS and his research is supported by the Austrian Science Fund (FWF) (PIN8074224) and the Horizon Europe project PoCCardio (grant number 101095432). DvL is supported by the Austrian Science Fund (FWF) Grant KLIF-1155-B.

**Data Availability** Data sharing is not applicable to this article as no data were created or analysed in this study.

## Declarations

**Competing interests** The authors declare no competing interests.



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# 7. Podsumowanie i wnioski

## 7.1. Podsumowanie

W ramach rozprawy doktorskiej połączono podejście kliniczne, molekularne oraz bioinformatyczne, uzupełnione metodami uczenia maszynowego, w celu zbadania roli sieci regulacyjnych ncRNA/sirtuin w zawale mięśnia sercowego oraz ich modulacji przez nowoczesne terapie kardiometaboliczne.

W badaniu klinicznym o charakterze translacyjnym, przeprowadzonym u pacjentów po ostrym zawale mięśnia sercowego, wykazano, że terapia empagliflozyną w porównaniu do placebo, wiąże się z istotnymi zmianami w ekspresji sirtuin oraz miRNA. Terapia empagliflozyną była związana ze wzrostem ekspresji SIRT6 i spadkiem ekspresji SIRT4, co wskazuje na wpływ na szlaki związane z funkcją mitochondrialną, stresem oksydacyjnym i przeżyciem komórkowym. Wykazano również, że wyjściowe poziomy SIRT2, SIRT4, miR-182-5p oraz miR-302a-3p u pacjentów w ostrej fazie po zawale mięśnia sercowego, są niezależnymi markerami predykcyjnymi zmiany frakcji wyrzutowej lewej komory. Panel biomarkerów obejmujący te cząsteczki wykazywał wyższą wartość predykcyjną niż pojedyncze markery, co wskazuje na ich potencjał w stratyfikacji pacjentów pod względem odpowiedzi na leczenie.

Druga praca wchodząca w skład cyklu publikacji rozszerzyła interpretację wyników części klinicznej poprzez analizę wspólnych sieci molekularnych związanych z inhibitorami SGLT2 oraz terapiami inkretynowymi. Wykazano, że nowoczesne terapie kardiometaboliczne działają w obrębie częściowo wspólnych szlaków regulacyjnych obejmujących procesy zapalne, metabolizm energetyczny, odpowiedź na stres komórkowy oraz funkcję układu sercowo-naczyniowego. Wyniki te wskazują, że działanie empagliflozyny należy interpretować w szerszym kontekście systemowych mechanizmów kardioprotekcyjnych.

Trzecia publikacja dostarczyła mechanistycznego tła biologicznego dla wyników uzyskanych w części klinicznej, podsumowując aktualną wiedzę dotyczącą roli ncRNA regulujących szlaki sirtuinowe w uszkodzeniu niedokrwienno-reperfuzyjnym mięśnia sercowego. Przegląd ten wykazał, że osie ncRNA-SIRT wpływają na kluczowe procesy związane z uszkodzeniem kardiomiocytów, w tym apoptozę, stres oksydacyjny, dysfunkcję mitochondrialną, pyroptozę i stan zapalny. Jednocześnie w związku z ograniczoną ilością danych pochodzących z badań klinicznych, wskazał na lukę w tym obszarze nauki.

Całość przedstawionych wyników wskazuje, że oś ncRNA-SIRT stanowi istotny element regulacji odpowiedzi mięśnia sercowego na uszkodzenie niedokrwienno-reperfuzyjne oraz może być modulowana przez empagliflozynę. Szlaki te integrują sygnały związane z metabolizmem, stanem zapalnym, funkcją mitochondriów i odpowiedzią na stres, wpływając na funkcję mięśnia sercowego po uszkodzeniu niedokrwienno-reperfuzyjnym. Z perspektywy translacyjnej, wyniki te wskazują na potencjał krążących ncRNA i SIRT jako biomarkerów predykcyjnych oraz narzędzi wspierających personalizację terapii, co może przyczynić się do rozwoju spersonalizowanych strategii terapeutycznych oraz poprawy rokowania pacjentów z chorobami układu sercowo-naczyniowego.

### 7.2. Wnioski

1. Terapia empagliflozyną u pacjentów po zawale mięśnia sercowego wiąże się z modulacją ekspresji wybranych sirtuin, w tym wzrostem ekspresji SIRT6 oraz obniżeniem ekspresji SIRT4.
2. Wyjściowe poziomy SIRT2, SIRT4, miR-182-5p oraz miR-302a-3p wykazują potencjał predykcyjny względem funkcji skurczowej lewej komory po 26 tygodniach leczenia empagliflozyną.
3. Łączny panel biomarkerów obejmujący SIRT2, SIRT4, miR-182-5p i miR-302a-3p charakteryzuje się najwyższą trafnością predykcyjną i może stanowić podstawę dalszych badań nad stratyfikacją pacjentów pod względem odpowiedzi na leczenie po zawale mięśnia sercowego.
4. Analizy bioinformatyczne wskazują, że nowoczesne terapie kardiometaboliczne, w tym inhibitory SGLT2, działają w obrębie sieci molekularnych związanych z metabolizmem, stanem zapalnym oraz odpowiedzią na stres komórkowy.
5. Oś ncRNA-SIRT może odgrywać istotną rolę w patofizjologii uszkodzenia niedokrwienno-reperfuzyjnego mięśnia sercowego i stanowi obiecujący obszar dalszych badań translacyjnych nad nowymi biomarkerami oraz potencjalnymi celami terapeutycznymi.
6. Integracja danych klinicznych, molekularnych i bioinformatycznych może wspierać rozwój spersonalizowanych strategii terapeutycznych u pacjentów po zawale mięśnia sercowego.

## 8. Piśmiennictwo


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## 9. Opinia Komisji Bioetycznej lub Etycznej

**TŁUMACZENIE PRZYSIĘGŁE Z JĘZYKA NIEMIECKIEGO**

*[Uwagi tłumacza podano w nawiasach kursywą]*

Ethikkommission Medizinische Universität Graz

Komisja Etyczna Uniwersytetu Medycznego w Grazu, Auenbruggerplatz 2, A-8036 GRAZ

Auenbruggerplatz 2, A-8036 Graz ethikkommission@medunigraz.at Tel.: +43 / 316 / 385-13928, Fax: -14348

POSTANOWIENIE ważne do 03.03.2018

Nr EK: 29-179 ex 16/17 Nr EudraCT: 2016-004591-22

Tytuł badania: Wpływ empagliflozyny na funkcję serca i markery niewydolności serca u pacjentów z ostrym zawałem mięśnia sercowego (EMMY-Trial) - badanie fazy III

Promotor / badający:], Klinika Chorób Wewnętrznych

Sponsor: Uniwersytet Medyczny Graz

Osoba kontaktowa sponsora:, Auenbruggerplatz 15, 8036 Graz

CRO:

Wnioskodawca: Univ. Klinik für Innere Medizin

Osoba kontaktowa wnioskodawcy: dr Norbert Tripolt, Auenbruggerplatz 15, 8036 Graz

Powyższe badanie zostało po raz pierwszy rozpatrzone przez Komisję Etyczną na posiedzeniu 04-16/17 w dniu 16.01.2017. Komisja etyczna doszła do następującego wniosku:

Nie stwierdzono zastrzeżeń do przeprowadzenia badania w przedstawionej formie.

Członkowie uprawnieni do głosowania lub obecni przy rozpatrywaniu: patrz załączona lista z dnia 16.01.2017.

Członkowie komisji, którzy byli uznani za stronniczych wobec tego punktu porządku obrad i w związku z tym nie brali udziału w podejmowaniu decyzji i głosowaniu: brak.

Dokumenty przedłożone do oceny: Dokumenty wpłynęły 23.12.2016, rozpatrzone na posiedzeniu 04-16/17 w dniu 16.01.2017

- Cover Letter - 23.12.2016
- Formularz zgłoszeniowy ECS - strony podpisów - 23.12.2016
- Formularz zgłoszeniowy ECS - 23.12.2016
- Oryginalny protokół Empagliflozin_Protocol V2.4 2.4 - 15.12.2016
- Formularz świadomej zgody Informed Consent Form V1.0 1.0 - 14.12.2016

- Case Report Form CRF V1.1 1.1 - 15.12.2016
- CV prof. Hoppe (PI Salzburg) - brak wersji - 19.12.2016
- CV Dirk von Lewinski - 22.12.2016
- CV Sourij - brak wersji - 16.03.2016
- Investigator's Brochure Investigator Brochure 14 - 15.07.2016

Dokumenty wpłynęły 27.12.2016, rozpatrzone na posiedzeniu 04-16/17 w dniu 16.01.2017:

- Wniosek Część B ECS podpisany, Centrum Hoppe - 23.12.2016





Dokumenty wpłynęły 04.01.2017, zaopiniowane na posiedzeniu 04-16/17 w dniu 16.01.2017

- Conflict of Interest - Oświadczenie Lewinski - 03.01.2017
- Conflict of Interest - Oświadczenie Sourij - 03.01.2017
- Formularz EudraCT (CT1) - Strona podpisów - 03.01.2017
- Inne: e-mail - Oświadczenie - 04.01.2017
- Inne: Certyfikat GCP, Sourij - 10.06.2015
- Inne: Umowa z Boehringer Ingelheim - projekt

Dokumenty wpłynęły 13.01.2017, zaopiniowane na posiedzeniu 04-16/17 w dniu 16.01.2017

- Cover Letter - 12.01.2017
- Formularz EudraCT (CT1) bez daty
- Inne: E-mail - Oświadczenie do punktów w formularzu zgłoszeniowym ECS - 13.01.2017

Dokumenty wpłynęły 31.01.2017 (zaopiniowane przy następnej ocenie)

- Oryginalny protokół 2.5 - 31.01.2017
- Inne: Informator dla lekarza rodzinnego/zespół rehabilitacyjny 1.0 - 31.01.2017
- Inne: Oświadczenie w odpowiedzi na zawiadomienie o przetwarzaniu - 31.01.2017

Dokumenty wpłynęły 10.02.2017 (zaopiniowane przy następnej ocenie)

- Formularz świadomej zgody - Centrum Sourij 1.1 - 31.01.2017

Dokumenty wpłynęły 14.02.2017, zaopiniowane w „expedited Review" (przyspieszonym trybie) w dniu 03.03.2017

- Zaświadczenie ubezpieczeniowe Wiener Städtische 08-N811.957 - 14.02.2017

Dokumenty wpłynęły 18.04.2017 (zaopiniowane przy następnej ocenie)

- Cover Letter - 14.04.2017
- Protokół - Amendment 1 - 14.04.2017
- Formularz świadomej zgody 1.1 - 14.04.2017
- Inne: Formularz zgłoszenia do EK - 14.04.2017

Dokumenty wpłynęły 27.04.2017, zaopiniowane w „expedited Review" w dniu 28.04.2017

- Oryginalny protokół 2.6 - 14.04.2017

Data pierwszego votum: 03.03.2017

Komisja Etyczna stwierdza - bez skutków prawnych - przyjmuje, że jest to badanie kliniczne w rozumieniu AMG i zwraca uwagę, że przed rozpoczęciem badania należy złożyć właściwy wniosek o zezwolenie do Federalnego Urzędu ds. Bezpieczeństwa w Ochronie Zdrowia (Bundesamt für Sicherheit im Gesundheitswesen).

Votum Komisji Etycznej w żaden sposób nie zwalnia wyłącznie badacza / badaczki z odpowiedzialności za prawidłowe przeprowadzenie badania zgodnie z obowiązującymi przepisami i wytycznymi.

Ponadto zwracamy uwagę, że komisji należy niezwłocznie zgłaszać:

- odstępstwa od protokołu z powodów bezpieczeństwa lub zmiany protokołu,
- zmiany, które zwiększają ryzyko dla uczestników/uczestniczek lub istotnie wpływają na przeprowadzenie badania,
- domniemane nieoczekiwane poważne niepożądane zdarzenia - SUSARy (studia AMG od 1.5.2004),
- każde inne zdarzenie lub okoliczność, które mogą mieć wpływ na bezpieczeństwo uczestników/uczestniczek lub na przeprowadzenie badania.

Termin ważności votum obejmuje ośrodki wymienione na liście z 03.03.2017.

EK-Nummer: 29-179 ex 16/17 Votum (28.04.2017) Strona 2 z 3

Medizinische Universität Graz, Auenbruggerplatz 2, A-8036 Graz, www.medunigraz.at

Forma prawna: osoba prawna prawa publicznego zgodnie z Universitätsgesetz 2002; informacje: Biuletyn informacyjny Uniwersytetu i www.medunigraz.at. DVR-Nr.: 210 9494. UID: ATU 575 111 79. Dane bankowe: Bank Austria Creditanstalt BLZ 12000, konto nr 500 948 400 04; Raiffeisen Landesbank Steiermark BLZ 38000, konto nr 49510.

Graz, 28 kwietnia 2017

(podpis)

Prof. univ. DI dr Josef Haas

Przewodniczący

(podpis)

Prof. univ. dr Hermann Toplak

Z-ca przewodniczącego

Uwaga: Prosimy we wszystkich pismach lub przy zapytaniach telefonicznych dotyczących projektu podawać numer EK!

Nr EK: 29-179 ex 16/17 Opinia (28.04.2017) Strona 3 z 3

Uniwersytet Medyczny w Graz, Auenbruggerplatz 2, A-8036 Graz, www.medunigraz.at

Forma prawna: osoba prawna prawa publicznego zgodnie z ustawą uniwersytecką z 2002 r.; informacje: Biuletyn Uniwersytetu i www.medunigraz.at. Nr DVR: 210 9494.

NIP (UID): ATU 575 111 79. Dane bankowe: Bank Austria Creditanstalt BLZ 12000, nr konta 500 948 400 04; Raiffeisen Landesbank Steiermark BLZ 32000, nr konta 49510.

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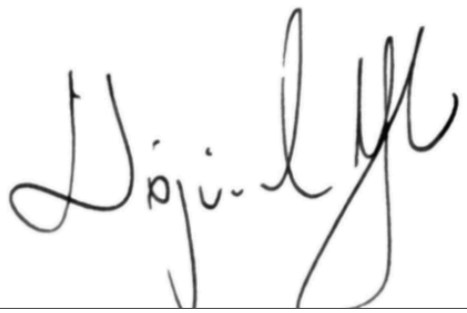
**REPERTORIUM NR 2354/2026**

Ja, Wojciech Szefler, tłumacz przysięgły języka niemieckiego (TP/3467/05), poświadczam niniejszym zgodność powyższego tłumaczenia z okazanym mi oryginalnym dokumentem sporządzonym w języku niemieckim.

Ciechanów, dnia 24.04.2026



**10. Oświadczenia wszystkich współautorów publikacji określające indywidualny wkład (udział merytoryczny i procentowy) każdego z nich w ich powstanie**

22/04/2026
(miejscowość, data)
Warszawa

Dr hab. n. med. Ceren Eyileten Postula

OŚWIADCZENIE

Jako współautor prac:
1) „*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: nadzór merytoryczny nad realizacją pracy, przygotowanie danych do analizy statystycznej, analiza statystyczna, przygotowanie pierwotnej wersji manuskryptu, opracowanie wizualizacji danych, krytyczną rewizję i redakcję manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 78%, obejmował on wykonanie analiz laboratoryjnych, przygotowanie danych do analizy, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu.

2) „*MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury*", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: nadzór merytoryczny nad realizacją pracy, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 94%, obejmował on przegląd literatury, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu.

3) „*Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health*", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: interpretację wyników, przygotowanie manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład lek. Anny Nowak-Szwed określam jako 20%, obejmował on zbieranie i przygotowanie danych oraz przygotowanie manuskryptu.

Wyrażam zgodę na wykorzystanie powyższych prac jako części rozprawy doktorskiej lek. Anny Nowak-Szwed.

Ceren Eyileten Postula
(podpis oświadczającego)

1/05/2026
(miejscowość, data)
Warszawa

Prof. dr hab. n. med. Marek Postuła

OŚWIADCZENIE

Jako współautor prac:

1) „*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", oświadczam, iż mój wkład obejmował nadzór merytoryczny nad realizacją pracy, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 78%, obejmował on wykonanie analiz laboratoryjnych, przygotowanie danych do analizy, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu.

2) „MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: nadzór merytoryczny nad realizacją pracy, przygotowanie pierwotnej wersji manuskryptu, krytycznę rewizję i redakcję manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 94%, obejmował on przegląd literatury, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytycznę rewizję i redakcję manuskryptu.

3) „Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: opracowanie koncepcji i projektu badania, interpretację wyników, nadzór nad przygotowaniem manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 20%, obejmował on zbieranie i przygotowanie danych oraz przygotowanie manuskryptu.

Wyrażam zgodę na wykorzystanie powyższych prac jako części rozprawy doktorskiej lek. Anny Nowak-Szwed.

Marek Postuła
(podpis oświadczającego)

Warszawa, 26.04.2026
(miejscowość, data)

Dr n. wet. Zofia Wicik

OŚWIADCZENIE

Jako współautor prac:

1) „*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: wykonanie analiz bioinformatycznych i analiz z wykorzystaniem uczenia maszynowego, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 15%.
Wkład lek. Anny Nowak-Szwed określam jako 78%, obejmował on wykonanie analiz laboratoryjnych, przygotowanie danych do analizy, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu.

2) „*MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury*", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: wykonanie analiz bioinformatycznych, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 94%, obejmował on przegląd literatury, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu.

3) „Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: opracowanie koncepcji i projektu badania, zbieranie i przygotowanie danych, przeprowadzenie analizy danych, opracowanie wizualizacji danych, interpretację wyników, przygotowanie manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 70%.
Wkład lek. Anny Nowak-Szwed określam jako 20%, obejmował on zbieranie i przygotowanie danych oraz przygotowanie manuskryptu.

Wyrażam zgodę na wykorzystanie powyższych prac jako części rozprawy doktorskiej lek. Anny Nowak-Szwed.

.....
(podpis oświadczającego)

Graz, 27APR2026
(place, date)

Prof. Dirk von Lewinski, MD, PhD

STATEMENT

As a co-author of the following papers:

1) "*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of data preparation, critical revision and editing of the manuscript.
The contribution of Anna Nowak-Szwed, MD is estimated at 78% and included performing laboratory analyses, data preparation, preparation of the original draft of the manuscript, and critical revision and editing of the manuscript.

2) "*MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of critical revision and editing of the manuscript.
The contribution of Anna Nowak-Szwed, MD is estimated at 94% and included literature review, development of data visualizations, preparation of the original draft of the manuscript, and critical revision and editing of the manuscript.

3) "*Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of the supervision of manuscript preparation.
The contribution of Anna Nowak-Szwed, MD is estimated at 20% and included data collection and preparation, as well as preparation of the manuscript.

I consent to the use of these works as part of the doctoral dissertation of Anna Nowak-Szwed, MD.

.....
(signature)

Gliwice, 25/APR/2026
(place, date)

Prof. Harald Sourij, MD, PhD

STATEMENT

As a co-author of the following papers:

1) "*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of data preparation, critical revision and editing of the manuscript.
The contribution of Anna Nowak-Szwed, MD is estimated at 78% and included performing laboratory analyses, data preparation, preparation of the original draft of the manuscript, and critical revision and editing of the manuscript.

2) "MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of critical revision and editing of the manuscript.
The contribution of Anna Nowak-Szwed, MD is estimated at 94% and included literature review, development of data visualizations, preparation of the original draft of the manuscript, and critical revision and editing of the manuscript.

3) "*Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of the supervision of manuscript preparation.
The contribution of Anna Nowak-Szwed, MD is estimated at 20% and included data collection and preparation, as well as preparation of the manuscript.

I consent to the use of these works as part of the doctoral dissertation of Anna Nowak-Szwed, MD.

.....
(signature)

Vienna, 30.04.2026
(place, date)

Prof. Jolanta Siller-Matula, MD, PhD

STATEMENT

As a co-author of the paper entitled "*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of critical revision and editing of the manuscript.

The contribution of Anna Nowak-Szwed, MD is estimated at 78% and included performing laboratory analyses, data preparation, preparation of the original draft of the manuscript, and critical revision and editing of the manuscript.

I consent to the use of this work as part of the doctoral dissertation of Anna Nowak-Szwed, MD.

Jolanta Siller-Matula
.....
(signature)

Warsaw, 24.05.2026
(place, date)

Dr Jeff Palatini

STATEMENT

As a co-author of the paper entitled "*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of critical revision and editing of the manuscript.

The contribution of Anna Nowak-Szwed, MD is estimated at 78% and included performing laboratory analyses, data preparation, preparation of the original draft of the manuscript, and critical revision and editing of the manuscript.

I consent to the use of this work as part of the doctoral dissertation of Anna Nowak-Szwed, MD.

Jeff Palatini
(signature)

23.04.2026
(miejscowość, data)

Mgr Sara Ahmadova

**OŚWIADCZENIE**

Jako współautor prac:

1) „*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", oświadczam, iż mój wkład obejmował wykonanie analiz laboratoryjnych. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 78%, obejmował on wykonanie analiz laboratoryjnych, przygotowanie danych do analizy, przygotowanie pierwotnej wersji manuskryptu, krytyczną rewizję i redakcję manuskryptu.

2) „*MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury*", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi przygotowanie pierwotnej wersji manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 94%, obejmował on przegląd literatury, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytycznę rewizję i redakcję manuskryptu.

Wyrażam zgodę na wykorzystanie powyższych prac jako części rozprawy doktorskiej lek. Anny Nowak-Szwed.

SARA AHMADOVA
(podpis oświadczającego)

Riga, 22.04.2026
(place, date)

Dr Svjatoslavs Kistkins

STATEMENT

As a co-author of the paper "*Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health*", I declare that my contribution to the preparation of the publication is estimated at 1% and consisted of the supervision of manuscript preparation.
The contribution of Anna Nowak-Szwed, MD is estimated at 20% and included data collection and preparation, as well as preparation of the manuscript.

I consent to the use of this work as part of the doctoral dissertation of Anna Nowak-Szwed, MD.

S. Kistkins
(signature)

Warszawa 04.05.2026
(miejscowość, data)

Dr Joanna Borkowska

OŚWIADCZENIE

Jako współautor pracy „*Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health*” oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: przygotowanie manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Anny Nowak-Szwed określam jako 20%, obejmował on zbieranie i przygotowanie danych oraz przygotowanie manuskryptu.

Wyrażam zgodę na wykorzystanie powyższej pracy jako części rozprawy doktorskiej lek. Anny Nowak-Szwed.

Joanna Borkowska
(podpis oświadczającego)

Warszawa, 25.04.2026
(miejscowość, data)

Lek. Anna Nowak-Szwed

OŚWIADCZENIE

Jako współautor prac:

1) „*Sirtuins and regulatory miRNAs as epigenetic determinants of empagliflozin-mediated recovery after acute myocardial infarction*", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: wykonanie analiz laboratoryjnych, przygotowanie danych do analizy, przygotowanie pierwotnej wersji manuskryptu, krytyczna rewizja i redakcja manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 78%.

2) „MicroRNAs and Long Non-coding RNAs Associated With Sirtuin Pathways in Myocardial Ischemia/Reperfusion Injury", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: przegląd literatury, opracowanie wizualizacji danych, przygotowanie pierwotnej wersji manuskryptu, krytyczna rewizja i redakcja manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 94%.

3) „Integrative gene–metabolite network analysis of GLP-1 receptor agonists and related incretin pathways in cardiometabolic health", oświadczam, iż mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: zbieranie i przygotowanie danych oraz przygotowanie manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako 20%.

Anna Nowak-Szwed

.....
(podpis oświadczającego)